THE ROLE OF REACTIVE OXYGEN SPECIES IN ~~T CELL~~ LYMPHOCYTE ACTIVATION.

A Thesis submitted by

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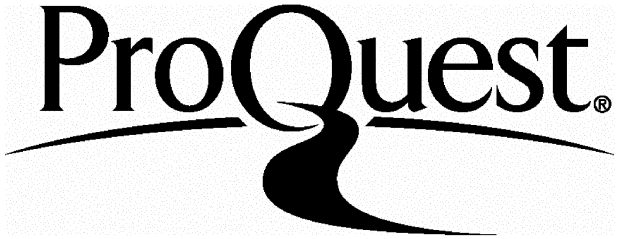

Abstract.

The exact nature of the biochemical events which follow T cell antigen recognition, in conjunction with the appropriate major histocompatibility complex, still remains incompletely understood.

It is well documented that oxygen free radicals are capable of oxidising various biological molecules leading to cellular alterations and if present in sufficient amounts, ultimately to cell death. Recently it has been suggested that smaller fluxes contribute to T cell effector functions.

The aim of the work was to study the role of reactive oxygen species (ROS) in T cell activation.

The effect of various stimuli (phorbol ester PMA, calcium ionophore A23187, monoclonal antibodies, anti-CD3, anti-CD28 and anti-CD2) on three lymphocyte functions, ie. thymidine incorporation (measure of lymphocyte proliferation), IL-2 release and ROS production were analysed. Thymidine incorporation and IL-2 release were measured 48 hours after the addition of stimuli. ROS production was measured using the free radical sensitive dye 2'7'-dichlorofluorescein by flow cytometry.

ROS fluxes, subsequent IL-2 release and proliferation were compared. The relationship between ROS and all responses was further examined using ROS scavengers (desferrioxamine, ascorbic acid, N-acetyl cysteine, vitamin E and DMSO) known to have differing modes of action. The concentration of antioxidant needed to block the enhancement of ROS production was compared to that needed to inhibit proliferation and IL-2 release by a similar degree.

The data suggest that the generation of ROS can be dissociated from the subsequent events of T cell activation, and provides support for the hypothesis that the redox status of the cell and not the free radicals themselves are the crucial factor determining gene activity in these cells.

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CHAPTER 5 - ROLE OF REACTIVE OXYGEN SPECIES IN THE T CELL ACTIVATION PATHWAYS STIMULATED BY ANTI-CD28 PLUS PMA OR ANTI-CD3

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Presentations and publications.

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Oxygen radical generation stimulated by PMA in synergy with anti-CD3.
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J.Immunol., (submitted).

Abbreviations.

Ag	Antigen
APC	Antigen presenting cell
ARAM	Antigen recognition activation motif
cAMP	cyclic Adenosine monophosphate
CD	Cluster of differentiation
CTLA	Cytolytic associated antigen
CTLL	Cytotoxic T lymphocyte line
CsA	Cyclosporin A
Cu	Copper
DAG	Diacylglycerol
DCF	2'7'-dichlorofluorescein
DCFH-DA	2'7'-dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e^-	Electron
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
Fe	Iron
FITC	Fluorescein isothiocyanate
G-protein	Guanine nucleotide binding protein
GAP	GTPase activating protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
H_2O	Water
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HBBS	Hank's balanced salt solution
HEPES	4-(-Hydroxyethyl)-1-piperazineethanesulfonic acid
Ig	Immunoglobulin
IL	Interleukin
IL-2 R	Interleukin-2 receptor
IP_3	Inositol (1,4,5)-triphosphate
IP_4	Inositol (1,3,4,5)-tetrakisphosphate
LFA	Leukocyte function associated molecule
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MAPK	MAP kinase

MAPKK	MAP kinase kinase
MEM	Minimum essential medium
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine diphosphate (reduced form)
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
NO•	Nitric oxide radical
O	Oxygen atom
O_2	Molecular oxygen (dioxygen)
O_2^-•	Superoxide radical anion
OH^-	Hydroxyl anion
OH•	Hydroxyl radical
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PI	Phosphoinositide
PIP_2	Phosphatidylinositol (4,5)-biphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PI-3- kinase	Phosphatidylinositol 3' hydroxyl kinase
PKC	Protein kinase C
PL	Phospholipase
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
r-IL-2	Recombinant Interleukin-2
RE	Responsive element
RPMI	Roswell park memorial institute
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SOS	Son of sevenless
TCR	T cell receptor
TNF	Tumour necrosis factor

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CHAPTER 1

INTRODUCTION

1.1 General introduction.

Evidence is growing that since cells continuously produce ROS, an intracellular level of ROS exists which maintains the normal redox status of the cell. Consequently it has been suggested that this redox status may play a major role in the fundamental cellular events of activation and that ROS could be potential candidates for the "missing" mediators of the complex signalling cascade. In this thesis the role of ROS in lymphocyte activation will be examined.

The analysis of signalling pathways from receptor ligation to activation of nuclear events remains incompletely understood. Here, T cell activation will refer to the events triggered by T cell antigen receptor stimulation, in conjunction with the appropriate major histocompatibility molecule leading to the production of cytokines and their receptors, with particular reference to interleukin-2.

The interaction of antigen with the specific receptor represents only the first step of a cascade of molecular events. This cascade of events is initiated by activation of intracellular protein tyrosine kinases crucial for all subsequent T cell responses. For example, one protein tyrosine kinase pathway involves regulation of G-proteins: *ras* being of special interest. In addition co-receptor engagement (eg. CD4 or CD8, CD2 and CD28) is vital for full T cell activation.

Although oxygen is vital for existence of eukaryotic aerobic forms of life, it can also be responsible for harmful cellular oxidative damage. This oxygen paradox is directly related to the chemical nature of the oxygen atom (O). The oxygen atom and its molecular form (O_2) both have unpaired electrons; hence, they are free radicals. The presence of these electrons permits ample opportunity for chemical reactivity. If these reactive oxygen species (ROS) are present in excess they can be a major cause of cellular damage and toxicity. Thus a balance between the flux of ROS and the efficiency of cellular protection mechanisms can determine cell survival. Ultimately, the quantity of ROS determines whether a cell suffers mild oxidant stress or cytotoxic oxidative damage. In recent years, ROS have been implicated in various pathologies including inflammation, cancer, neurogenerative diseases and ageing. Work involved

in this area has been vast, involving the study of the detrimental effects of ROS at the tissue, cellular and molecular level. Multiple mechanisms exist whereby oxygen radicals can cause cell injury; these range from protein damage, DNA fragmentation, via induction of breaks in single and double-stranded DNA, to lipid peroxidation resulting in the loss of membrane fluidity and maintenance of intracellular ionic gradients leading ultimately to cellular lysis. The existence of a specialised antioxidant system is vital, since ROS are produced not only as cytoxic agents but by every living cell as side products of electron transfer reactions in aerobic respiration. The antioxidant system comprises a variety of water and lipid soluble antioxidants as well as a series of antioxidant enzymes.

Although the detrimental effects of ROS are well documented and the role of oxidative signalling is rapidly emerging, little information regarding possible redox-sensitive "targets" is available. The identification of regulatory proteins for oxidative signalling would greatly advance the role of ROS in T cell activation.

1.2 Signal Transduction.

Resting T cells are in G_0 of the cell cycle. Activation is initiated by the interaction between an antigen associated with a membrane-bound major histocompatibity complex (MHC) molecule, presented on the surface of an antigen-presenting cell, and the T cell antigen receptor. This is sufficient for cell cycle entry (G_0 to G_1 phase transition) and the expression of high affinity receptors for T cell growth factors, in particular IL-2 (Abraham *et al.*,1992). Interaction of IL-2 with the IL-2 receptor then drives the progression of T cells from the G_1 phase to S phase and DNA synthesis (Cantrell *et al.*,1984). IL-2 and IL-2 receptor synthesis requires gene activation, messenger RNA transcription and protein synthesis (Isakov *et al.*,1986). Hence mechanisms must exist whereby intracellular biochemical signals in the form of "second messengers" transduce information originating at the cell surface to specific targeted genes in the nucleus. The exact nature of the biochemical events occurring between cell stimulation and gene activation is still unclear.

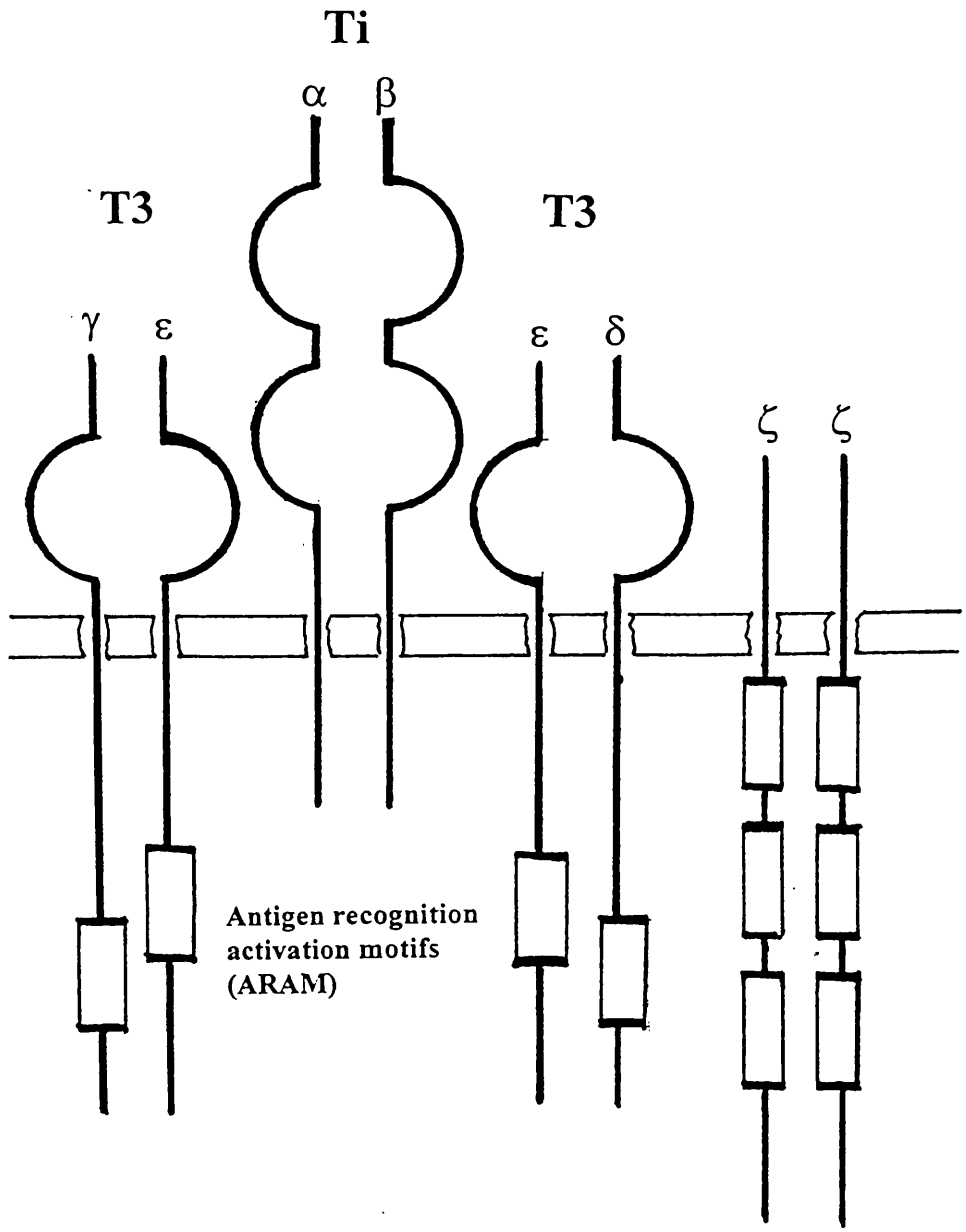
1.2.1 The T-cell receptor- CD3 complex.

An interaction between the antigen and the T cell receptor is vital for T cell activation. The T-cell receptor (TCR) has two main functions, it must first recognise and bind the antigen in the context of the appropriate MHC molecule, then translate this binding event into intracellular signalling by means of second messengers, ultimately resulting in adequate cellular responses.

The TCR is a complex structure (see Figure 1.1) consisting of six different polypeptides, which can be divided into three distinct subgroups of proteins (Klausner *et al.*,1991).

Figure 1.1

Structure of the TCR.



		molecular weight			molecular weight
Ti	α	43-54 kDa	T3	δ	22kDa
	β	38-44kDa		ε	21kDa
				γ	26-28kDa

The two clonally variable TCR chains, Ti α and β, are transmembrane proteins that span the bilayer once and have small intracytoplasmic domains of less than twelve amino acids which makes them unsuitable for transmembrane signalling (Weissman, 1994). The α and β chains together define the antigen specificity of the TCR (Chan *et al.*,1992a). Non-covalently associated with this disulphide-linked αβ dimer are three additional transmembrane proteins (ε, γ, δ) that form the CD3 or T3 complex (Finkel *et al.*,1991). In contrast to the Ti chains, these contain extracellular immunoglobulin like domains with larger cytoplasmic domains (Chan, 1994). Further studies have identified additional CD3 chains (ζ homodimer and a disulphide linked ζ_n heterodimer) but these are generally considered to be subunits independently associated with the TCR rather than actual components of the CD3 complex (Weiss and Imboden, 1987). The CD3 complex is responsible for transmembrane signal transduction following antigen binding to αβ (Malissen *et al.*,1993). The efficiency of surface expression is dependent on the assembly of all subunits of the TCR, implying that each subunit plays an integral role in the normal functioning of this receptor (Abraham *et al.*,1992).

The third subunit is the ζ family of hetero- and homodimers, which have only a short extracellular domain of nine amino acids (Weissman *et al.*,1994). This family of dimers has been found to be genetically and structurally distinct from CD3 (Chan *et al.*,1992; Klausner *et al.*,1991). The individual functions of the different chains of the TCR complex are not known. However, studies have provided direct evidence for a functional importance of the ζ chain in antigen-stimulated signalling, showing that cross-linking of the ζ chain was sufficient to cause intracellular calcium and IL-2 release (Irving *et al.*, 1993; Weiss, 1987). Subsequently, it was found that the TCR is composed of at least two signalling moieties (Chan *et al.*,1992). The ζ family dimer and the CD3 complex are thought to generate structurally distinct cellular responses (Weissman *et al.*, 1988). Evidence in support of this comes from examining the functional domains of the ζ chain and CD3. Antigen recognition activation motifs (ARAM) are tyrosine- and leucine-dependent sequence motifs, responsible for signal

transduction through these chains. On examination, it was found that the ARAM was triplicated in ζ and present as a single copy in each of the CD3 chains (refer back to figure 1.1) (Weiss, 1993). Cross-linking with anti-CD3ε or ζ resulted in different patterns of protein tyrosine phosphorylation, thus suggesting that distinct ARAMs within the TCR influence different signal transduction molecules. Alternatively, each module may couple to a common pathway and thus be important in amplifying the initial signal (Foster, 1993). Although the exact significance of these distinct signalling capabilities remains unclear, their importance in differential cytokine secretion and in determining cell activation or anergy should not be under estimated (Chan *et al.*,1994).

The use of monoclonal antibodies reactive with CD3 has facilitated a greater understanding of its role in T cell activation. Mitogen or growth factor activation of quiescent or undifferentiated cells results in rapid changes in the intracellular concentration of several cations, in particular calcium (Oettgen *et al.*,1985). Numerous studies have shown that the T cell mitogen phytohemagglutinin (PHA) produces an increase in calcium detectable within two minutes of addition (Mills *et al.*,1985; Scanlon, 1987; Tsein *et al.*,1982). Development of fluorescent calcium indicators, Quin-2 and Fura, have greatly facilitated the study of T cell calcium fluxes (Rabinovitch *et al.*,1986; Tsein *et al.*, 1988; 1982a). The calcium ionophore, A23187 was also found to be mitogenic (Akerman and Anderson, 1984). Subsequently, monoclonal antibodies reactive with the T3 complex (eg. UCHT-1, OKT3, Leu-4) have been shown to stimulate an increase in cytoplasmic free calcium (O'Flynn *et al.*,1985; Weiss *et al.*,1984). The TCR/CD3-mediated change in intracellular calcium is not affected by organic calcium channel blockers, suggesting that classical voltage-operated calcium channels are not involved (Imboden *et al.*,1985a). While calcium is a necessary signal, it is not sufficient for the full process of activation, ie proliferation, and an additional secondary signal is needed.

1.2.2 Tyrosine phosphorylation and protein tyrosine phosphatases.

The earliest biochemical event detected following TCR engagement is tyrosine phosphorylation (June *et al.*,1990). The TCR is composed of multiple subunits none of which contain intrinsic tyrosine kinase activity, unlike other receptors eg. the epidermal growth factor receptor.

TCR stimulation induces protein tyrosine kinase (PTK) activation, resulting in the tyrosine phosphorylation of multiple cellular proteins. Evidence in support of this has come from the use of tyrosine kinase inhibitors (eg. herbimycin A), which inhibit both inositol phosphate turnover and IL-2 and IL-2 receptor expression. These experiments provide a direct indication that PTK activation is an early event vital to the cellular activation induced by TCR.

Tyrosine phosphorylation is regulated by two antagonistic group of enzymes, protein tyrosine kinases and protein tyrosine phosphatases (PTP). Lymphocytes express several intracellular and transmembrane PTP. The best characterised PTP in lymphocytes is CD45, which is essential for TCR function and PTK regulation (McFarland *et al.*, 1994).

A. Tyrosine Kinases.

TCR stimulation results in the phosphorylation of tyrosines within the ARAM. It has been speculated that different ARAMs can interact with distinct PTKs (Sexton *et al.*, 1994). The three main PTKs implicated in TCR function are fyn (59kDa), lck (56kDa) and ZAP-70 (70kDa). These can be structurally sub-divided; fyn and lck are members of the src family of kinases and ZAP-70 belongs to the syk family.

(i) *fyn.*

Two isoforms of fyn have been identified, fynB, expressed in the brain and fynT in hematopoietic cells (Semba *et al.*,1991). These isoforms differ in their SH_2 sequence homology domains, which are responsible for mediating interactions with other proteins and regulating substrate access to the catalytic domains of the src family of

PTKs (Rudd *et al.*,1994). The catalytic function of fyn is thought to be stimulated by the engagement of the TCR by antigen / MHC (Peri *et al.*,1994).

Much evidence exists for the involvement of fyn in TCR function. Over-expression of fyn in thymocytes results in enhanced signalling, with respect to calcium flux and proliferation (Cooke *et al.*,1991; Weissman, 1994). Other studies have demonstrated that the expression of an inactive fyn can have negative effect on signalling (Chan *et al.*,1992). The exact nature of the TCR-fyn complex remains unclear but it is suggested that fyn(T) can interact with the TCR ζ chain and the CD3 complex through its N-terminal region (Rudd *et al.*,1994).

(ii) *lck.*

lck expression is restricted to lymphoid cells. lck has been shown to be non-covalently associated with the cytoplasmic domains of two T cell surface antigens, CD8 and CD4, which recognise class I and II MHC determinants on antigen presenting cells respectively (Veillette *et al.*,1988). CD4 / CD8 binding to MHC molecules stabilises the interaction between T cells and antigen-presenting cells, thus making them important co-receptors in signal transduction (Chan *et al.*,1992; Katayama *et al.*,1993). In addition, CD4 and CD8 allow MHC-dependent association of activated lck molecules with the TCR. Numerous studies on lck have shown that it plays a critical role in the signalling of T cell activation (Peri *et al.*,1994).

In addition to CD4 and CD8, other regulatory T cell surface molecules which may be important in lck regulation include the β-chain of the IL-2 receptor, CD2 and CD45. The importance of CD45 regulation of lck will be discussed in section 1.2.2.B.

(iii) *ZAP-70.*

ZAP-70 is a member of the syk family of kinases. It binds to phosphorylated ζ chains and CD3 components (Fraser *et al.*,1993). TCR-ζ and ZAP-70 association is dependent on lck or fyn(T) (Chan, 1994). It is thought that either lck or fyn initiates activation by phosphorylating one or more tyrosines in the ARAM of ζ or CD3, allowing subsequent ZAP-70 binding. Since ZAP-70 is a tyrosine kinase it has the

potential to modulate the TCR-induced tyrosine phosphorylation. In addition, the existence of multiple ARAMs within the TCR complex means that many ZAP-70 molecules can co-localize with the TCR complex, thus amplifying the initial signal (Mallissen *et al.*,1993).

Recent evidence has implicated another important member of the src-like kinase family, csk (50kDa). This cytoplasmic kinase has been suggested to have an involvement in the negative regulation of T cell activation. Its exact mode of action is unclear, but it could be involved in the physiological termination of TCR- mediated T cell activation (Cloutier *et al.*,1995). It is proposed that csk may phosphorylate a negative regulatory carboxy terminal site of lck and fyn. Furthermore csk has been speculated to phosphorylate CD45, increasing its phosphatase activity and affecting its association with lck (Autero *et al.*,1994).

B. CD45 and lck regulation.

CD45 is a protein tyrosine phosphatase (PTP), found in nucleated cells of the hematopoietic system. Transmembrane protein tyrosine phosphatases have an extracellular domain, a single transmembrane domain and a cytoplasmic region containing the catalytic domain (Weaver *et al.*, 1992).

PTP are antagonistic to PTK since they dephosphorylate tyrosine phosphoproteins. Paradoxically CD45 is vital for T cell activation. CD45-deficient cells are unable to secrete IL-2 or proliferate following TCR stimulation (Weaver *et al.*, 1991). In contrast, the use of CD45-deficient cells, still respondant to IL-2, has shown that IL-2 receptor signal transduction is independent of CD45 (Cantrell *et al.*,1993).

CD45 associates with a number of T cell surface molecules including the TCR, co-receptors CD4 and CD8, and CD2 (Chan *et al.*,1994). Co-ligation of the stimulatory CD2 molecule with CD45 has a marked inhibitory effect on T cell activation (Samelson *et al.*,1990). How the CD2 utilises the TCR for signalling is unclear. The degree of CD45 receptor saturation is thought to be an important determinant of the positive or negative regulatory effect of CD45 on early signals (Ledbetter *et al.*,1993).

The importance of lck in TCR activation is emphasised by its interaction with CD45. All src family members have two regulatory tyrosine residues. In lck these are tyr394 and tyr505. Tyr394 is an autophosphorylation site, important in increasing tyrosine kinase activity. Tyr505, the negative regulatory site, has been shown to be the predominant phosphorylated form in CD45-deficient cells (Chan *et al.*,1992). Thus, CD45 dephosphorylation of tyr505 may be responsible for increasing activated PTK activity for TCR signalling (Ledbetter *et al.*,1993).

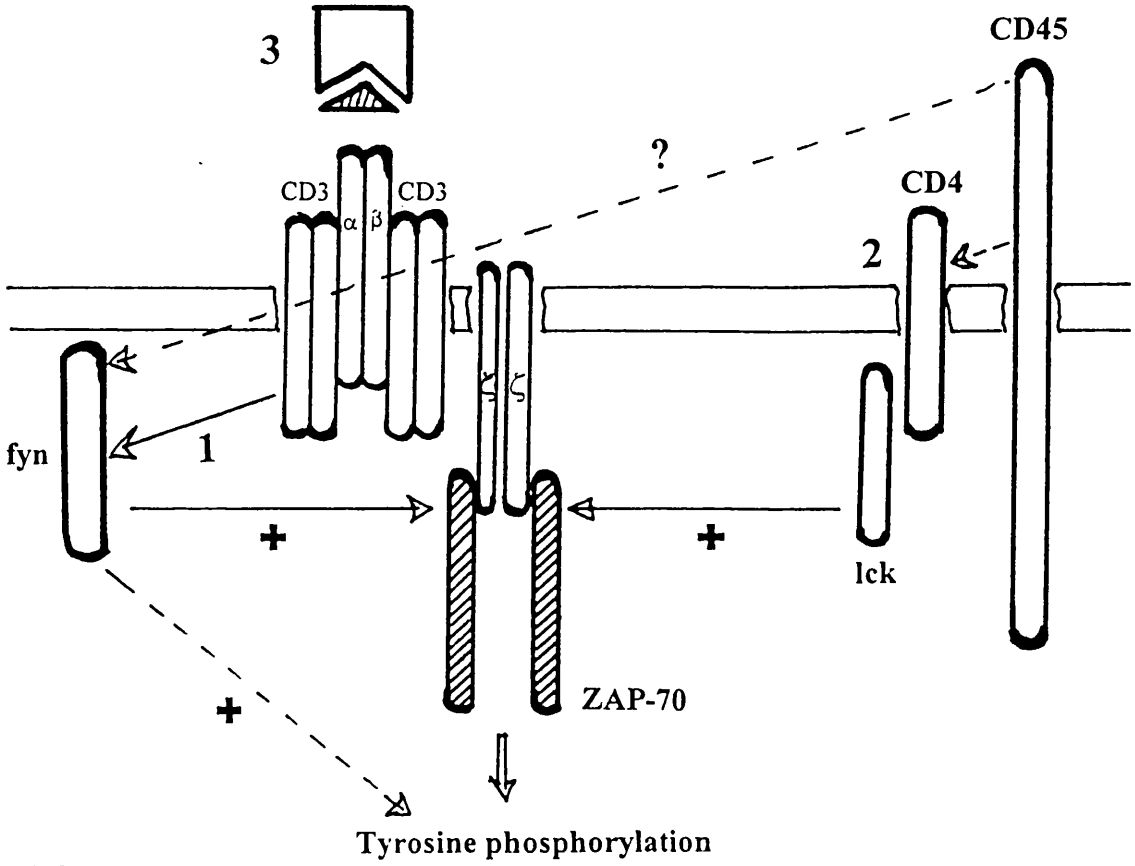
C. Overall model of PTK and PTP regulation.

The exact functional role of the three non-receptor tyrosine kinases and the tyrosine phosphatases in T cell receptor signalling is not completely understood. Whereas ZAP-70 and fyn(T) become physically associated with the T cell antigen receptor, lck appears to be essential for TCR signalling without directly being coupled to the TCR (Tsygankov *et al.*,1994). A proposed model is outlined in Figure 1.2.

Figure 1.2

Proposed model for PTK and PTP regulation.

Adapted from Klausner *et al.*, 1991.



KEY.

1. At the surface of the T cell, the TCR is associated directly/ indirectly with fyn and

2. lck is associated with CD4.

3. Engagement of the TCR with antigen (Ag) bound to the appropriate MHC facilitates the interaction of fyn and lck with CD45. Interaction with MHC means that the CD4-lck pair are brought into a multimolecular complex with the TCR. This allows either, or both kinases to become activated. The two kinases could phosphorylate each other in such a complex, resulting in either an increased or decreased activity depending on the phosphorylation site (Klausner, 1991). The existence of multiple subdomains, means that fyn and lck can regulate a variety of signals, including IL-2 production and the release of intracellular calcium (Rudd *et al.*, 1994).

In summary, the TCR- induced signal involves the participation of enzymes involved in signal initiation (fyn(T) and lck), signal amplification (ZAP-70) and signal inhibition/ termination (csk).

It is well documented that PTKs couple the TCR to signalling pathways, one of which regulates inositol metabolism and another involves guanine nucleotide binding proteins. These pathways will be discussed below.

1.2.3. Inositol phospholipid metabolism.

A. Phospholipase C.

One of the earliest components to be detected in the PTK pathway is the phosphatidylinositol (PI)-specific phospholipase C-γ 1 (PLC-γ 1). PLC-γ 1 is a member of one of three major PLC family members. Tyrosine kinases phosphorylate this isoenzyme on at least three tyrosines and this is essential for activating the enzyme (Weiss *et al.*, 1991). Phosphoinositides are unique in comparison to other membrane phospholipids in that they can be phosphorylated at multiple sites (Berridge, 1987). PLC-γ 1 cleaves the phosphodiester bond between glycerol and phosphate in membrane phosphoinositides, in particular phosphatidylinositol 4,5 biphosphate (PIP_2). This hydrolysis requires the addition of a hydroxyl group (eg. as a free OH^-), generating two intracellular messengers, inositol (1, 4, 5)-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 mobilises calcium and DAG activates protein kinase C (PKC). These two intracellular messengers trigger two parallel pathways which act in concert to generate a physiological response (Isakov *et al.*,1986). The mode of action and the metabolism of IP_3 and DAG will be discussed first and this will be followed by a discussion of the synergy between calcium and PKC.

(i) Inositol (1, 4, 5)-triphosphate (IP_3).

IP_3 is a water soluble second messenger which plays a central role in T cell activation through its regulation of intracellular calcium (Berridge, 1993). IP_3- mediated release of calcium causes an initial peak in calcium levels lasting only a few minutes (Weiss

et al.,1987). The initial rise in the intracellular free calcium level is due to the mobilisation of intracellular calcium stores, but cannot explain the secondary sustained elevation of calcium, vital for cellular activation.

Initially, IP_3 binds to receptors within the endoplasmic reticulum to stimulate an efflux of calcium from these stores. These receptors have binding sites for IP_3, calcium and other factors located on large N-terminal regions which extend into the cytoplasm. The release of calcium is determined by the appropriate binding of IP_3 and calcium to the IP_3 receptor (Berridge, 1995; Taylor *et al.*, 1992). The secondary phase of increase in intracellular calcium is attributed to IP_3 receptors in the plasma membrane. A family of IP_3 receptors and their structures have recently been identified. The human type 1 IP_3 receptor (skeletal muscle-like) has been shown to undergo tyrosine phosphorylation during T cell activation (Harnick *et al.*,1995). Plasma membrane IP_3 receptors are thought to increase calcium entry through calcium-release activated calcium channels when the internal stores have been drained by IP_3 (Berridge *et al.*,1995). Monoclonal antibodies against CD3 and CD2 and the mitogen PHA have all been shown to generate a calcium flux by activating a common calcium channel (Gardner *et al.*,1989).

IP_3 metabolism is complex. It can be dephosphorylated to free inositol, which can be recycled into phospholipid, or it can be converted to inositol polyphosphates which may have second messenger function of their own (Weiss, 1989). Furthermore, IP_3 can be converted to IP_4 (inositol (1,3,4,5)-tetrakisphosphate) by a calcium-dependent cytoplasmic kinase. IP_4 has been shown to have important messenger functions, including the activation of calcium channels in the plasma membrane (Hansen *et al.*, 1988).

(ii) 1,2-Diacylglycerol.

Following PIP_2 hydrolysis, DAG remains within the membrane and functions as a second messenger by activation of protein kinase C (PKC). PKC is a calcium-dependent enzyme, composed of two functional domains; a hydrophobic domain which upon activation binds to the cell membrane, and a hydrophilic domain

containing the catalytic centre. DAG increases the affinity of PKC for calcium allowing activation to occur at calcium concentrations within the intracellular range. PKC activation results in the formation of a quaternary complex of PKC, DAG, phospholipid (phosphatidlyserine is the most efficient) and calcium (Clark and Ledbetter, 1986). Calcium is thought to cause the translocation of PKC from the cytosol into the membrane where it can be activated (Wolf *et al.*,1985). Activated PKC is responsible for the phosphorylation of numerous proteins important in T cell activation: these include the IL-2 receptor and transferrin receptor (Weiss, 1987). However, the exact implications of these phosphorylations is still unclear.

Initial production of DAG from PIP_2 has been shown to be only transient, corresponding to IP_3 formation. The initial peak is then followed by a more sustained DAG production due to the hydrolysis of phosphatidylcholine (another major structural block of the membrane bilayer) (Asaoka *et al.*,1992).

Recent studies have suggested that cis-unsaturated fatty acids such as arachidonic, oleic and unoleic acid, greatly enhance PKC activation. Fatty acids are products of the hydrolysis of phospholipids by the receptor-mediated activation of the enzyme phospholipase A_2 (PLA_2) (see below). Cis-unsaturated fatty acids increase the affinity of PKC for calcium, allowing it to exhibit almost full activation at nearly basal levels of calcium concentration (Shinomura *et al.*,1991). Consequently, PKC forms an integral part of the signal-induced activity of metabolic products of membrane phospholipids via the actions of phospholipases, which can no longer be considered as a simple process involving only the formation of IP_3 and DAG (Liscovitch, 1992).

DAG can be metabolized in two ways, either by phosphorylation to phosphatidic acid by a DAG kinase, or by hydrolysis by DAG lipase generating monoacylglycerol which is further hydrolysed to arachidonic acid.

Arachidonic acid.

Arachidonic acid can be derived from either the action of PLA_2 on phosphatidyl choline or from DAG metabolism. It has recently been found that the cytosolic 110kDa PLA_2 contains a calcium/ phospholipid binding domain which is sensitive to

elevated calcium levels and is responsible for the translocation of the enzyme from the cytosol to the membrane. In the membrane, it can be activated by PKC or a G-protein (Lisovitch, 1992). Arachidonic acid can be metabolised by two distinct oxidative pathways: the cyclooxygenase and lipoxygenase pathways. The enzyme cyclooxygenase converts arachidonic acid into endoperoxides which yield a variety of products, including thromboxanes and prostaglandins. Cyclooxygenase inhibitors have been shown to display little or no effect on T lymphocytes (Farrar *et al.*,1985). In contrast, great interest has grown in the lipoxygenase pathway which is responsible for generating a family of hydroxyeicosatetraenoic acids that are subsequently metabolised to leukotrienes. Lipoxygenase inhibitors can prevent mitogen-induced IL-2 production (Hadden, 1988).

(iii) Calcium and protein kinase C.

The activation of PKC and elevation of cytosolic calcium operating as a pair of parallel and synergistic mediators of T cell activation is a well studied stimulus (Nishizuka, 1983). These two synergistically acting pathways can be dissociated and studied separately by using agents to activate only one of them. Calcium ionophores (eg. A23187) or monoclonal antibodies directed against the CD3 complex can increase calcium. PKC activation has relied on the use of phorbol esters. The effect of these tools on T cells will be discussed separately in the context of their combined ability to bypass transmembrane signalling.

The diverse biological effects of phorbol esters (eg. phorbol 1,2 myristate acetate, PMA) have been attributed to their structural similarity to DAG, the physiological activator of PKC. In mammalian tissues, there are presently ten identified subspecies of PKC (Asaoka *et al.*,1992), although not all cells contain all the different isotypes. The biology of PKC could be far more complex than previously thought, since different subtypes respond differently to various combinations of calcium, DAG and phospholipid products. Consequently, it is not surprising that different types of phorbol esters (eg. PMA or phorbol dibenzoate) exert varying effects on T cells (Dobbs and Katz, 1987).

Despite the use of phorbol esters in numerous in vitro studies, there are limitations in their use. Phorbol esters bind to more than one species of PKC. Also its possible that in addition to activating PKC they may directly stimulate other signalling pathways. It is also necessary to be aware of the slow metabolism of phorbol esters in comparison to DAG. The phorbol ester translocation of PKC to the membrane is irreversible, whereas receptor-mediated activation of PKC can be transient (Weiss *et al.*,1987). Phorbol esters can induce stimulation of resting human T cells, in the presence of exogenous IL-2. However, alone they are unable to initiate the transcription of the IL-2 gene or cause IL-2 release.

The role of calcium in signalling is complex. It is implicated as a potential second messenger in T cell activation. One of its functions is to activate the early genes responsible for T cell progression from G_0 to the G_1 phase of the cell cycle (Berridge, 1995). Previously, it was thought that the regulatory effects of calcium on T cell activation were mediated by calcium-calmodulin-dependent kinases. Recently, the importance of calcineurin, a calcium-calmodulin-activated phosphatase, as the key signalling enzyme in T lymphocyte activation has been recognised (Clipstone and Crabtree, 1992). Calcineurin is composed of two subunits, a catalytic and a regulatory one. A rise in intracellular calcium activates calcineurin which acts in concert with PKC to stimulate the expression of the IL-2 gene (Lewis and Cahalan, 1995).

Calcium ionophores raise intracellular calcium by noncovalently binding to calcium ions and then carrying them through the cytoplasmic membrane (Reed, 1972). Much evidence exists for an increase in intracellular calcium following anti-CD3 binding (June, 1992). In addition, soluble monoclonal antibodies to CD3 and calcium ionophores display similar requirements in their abilities to activate T cells. Neither are effective alone, but both A23187 (Truneh *et al.*,1985; Procopio *et al.*, 1988) and anti-CD3 (Weiss *et al.*,1984) require a second additional signal, supplied by PMA, for lymphokine generation.

B. PI-3-kinase.

As mentioned in section 1.2.3.A, one pathway for the metabolism of inositol lipids, in particular PIP_2, is $PLC_{\gamma 1}$ which liberates IP_3 and DAG. Another pathway of inositol lipid metabolism originating from TCR activation is controlled by phosphatidylinositol 3' hydroxyl kinase (PI-3-kinase). PI-3 kinase catalyses the phosphorylation of phosphoinositides at the 3 hydroxyl position of the inositol ring, generating phosphatidylinositol triphosphate (PIP_3) (Foster, 1993). See Figure 1.3. PI-3 kinase is activated in response to both TCR and IL-2 receptor stimulation (Cantrell *et al.*,1993). It is composed of two subunits of 85 and 100kDa; the catalytic subunit is contained in the latter. The p85 subunit is important in recruiting the catalytic domain to PTKs and the plasma membrane.

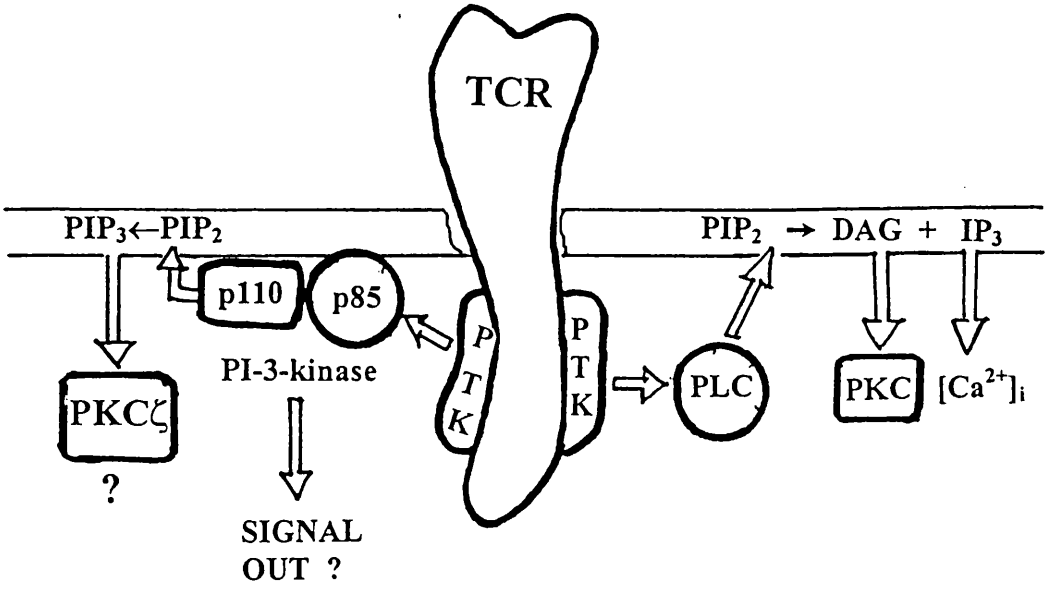
In vitro studies have shown that PIP_3 can stimulate an isoform of the PKC family. This recently identified isoform contains two atypical PKC subspecies: ζ, activated by PI-3-kinase and λ . The PI-3-kinase activated isoform is not sensitive to calcium, DAG or phorbol esters and is thought to be important in NF-κB activation (Ward *et al.*,1996). However, the exact implications of this finding remains to be determined.

The role of PI-3-kinase in T cell activation is attracting great interest. Its accumulation has been demonstrated following ligation of several T cell surface molecules. These include the T cell receptor-CD3 complex and CD2 (Ward *et al.*, 1992). It has also been speculated that PI-3-kinase activity may explain the synergy between CD28 and the TCR (Ward *et al.*,1996). The role of CD28 will be discussed in section 1.2.6.A.

Figure 1.3

TCR regulation of inositol lipid metabolism.

Taken from Cantrell, 1994.



Key.

TCR T cell receptor

PTK Protein tyrosine kinase

PKC Protein kinase C

PLC Phospholipase C

PIP_2 Phosphatidylinositol (4,5)-biphosphate

PIP_3 Phosphotidylinositol (3,4,5)-triphosphate

DAG 1,2- diacylglycerol

IP_3 Inositol (1,4,5)-triphosphate

$[Ca]_i$ Intracellular calcium concentration

1.2.4 G-proteins.

The term G-proteins refers to guanine nucleotide binding proteins. The G-proteins are part of a larger superfamily of proteins which have intrinsic GTPase activity. There are two main types of G-proteins; the heterotrimeric G-proteins which are composed of three distinct subunits α, β, and γ, and the low molecular weight monomeric proteins (Gilman, 1987). The low molecular weight *ras* family is best documented for its role in lymphocyte signalling.

A. Ras regulation.

The three members of the *ras* gene family Ha, Ki and N-ras encode 21 kDa molecular mass GTP binding proteins. These proteins have long been recognised for their regulatory role in growth and development (Marshall, 1995). *Ras* proteins function as signal transducers because of their ability to switch from an active to an inactive state in a guanine nucleotide binding cycle (Harnett and Rigley, 1992). Their intrinsic GTPase activity catalyses the hydrolysis of GTP (active form) to GDP, reactivation is then achieved by exchange of bound GDP for GTP (Hall, 1990). The guanine nucleotide binding cycle can be triggered by either the TCR or IL-2 receptor-coupled protein tyrosine kinases, resulting in the rapid and prolonged accumulation of active *ras*-GTP complexes (Pastor *et al.*, 1995). To date, there are two intracellular co-existing mechanisms for *ras* regulation in T cells, one of which is mediated by PKC. PKC activators and DAG induce rapid *ras* activation (Izquierdo *et al.*, 1990) which cannot be identified in other cells, such as fibroblasts and mast cells, implying that this PKC-mediated route may be restricted to T cells.

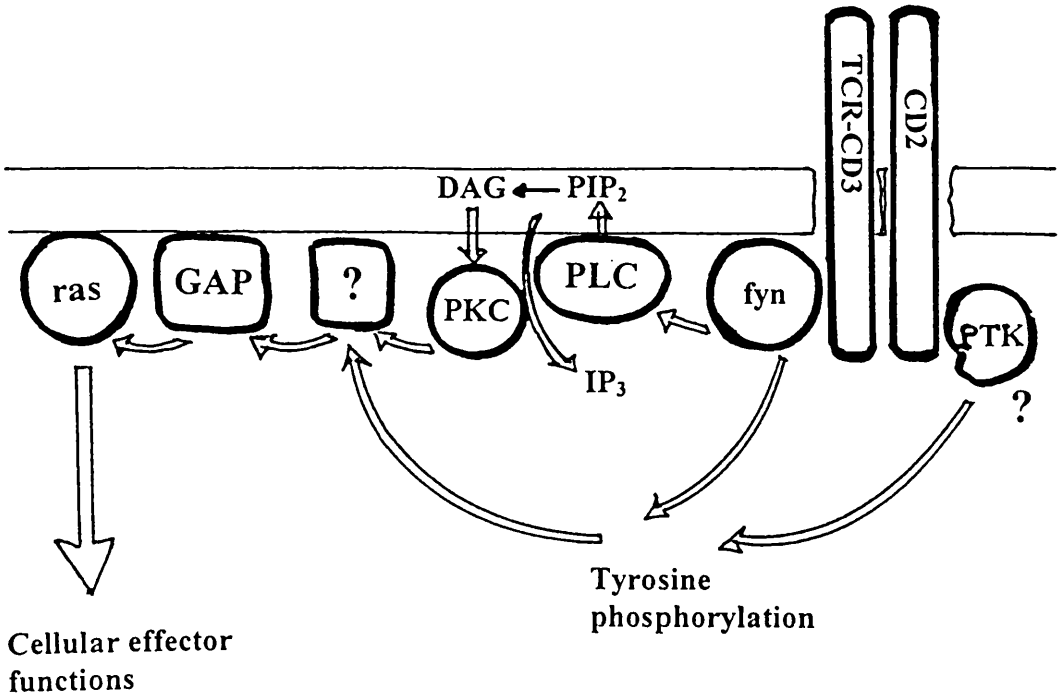
The precise molecular details of the alternative *ras* control mechanism are unknown, although tyrosine kinases are thought to be involved (Downward *et al.*, 1992). IL-2 receptor-mediated *ras* regulation is thought to occur via this second pathway, since it does not involve the activation of PKC (Cantrell *et al.*, 1992). Furthermore, CD2 which has been shown to induce *ras* activation (Graves *et al.*, 1991) could also be

acting via this non-PKC mediated pathway, although the possibility that it could be acting via both cannot be excluded (Figure 1.4).

Figure 1.4

Proposed model for CD2 regulation of *ras*.

Taken from Downward *et al.*, 1992.



Key.

PKC	Protein kinase C
PTK	Protein tyrosine kinase
PLC	Phospholipase C
PIP_2	Phosphatidylinositol (4,5)-biphosphate
DAG	1,2-diacylglycerol
IP_3	Inositol (1,4,5)-triphosphate
GAP	GTPase activating protein
?	novel protein

The sequence of events leading from TCR activation to *ras* has been studied in great detail, and does not appear to require PKC. Positive regulation of *ras* requires factors that catalyse guanine nucleotide exchange on *ras*. Guanine nucleotide exchange proteins characterised in mammalian cells include the homologue of the Drosophila "son of sevenless" (SOS) gene product. Other exchange proteins include a protooncogene product, Vav, which is a substrate for TCR stimulated PTKs (DeFranco, 1994).

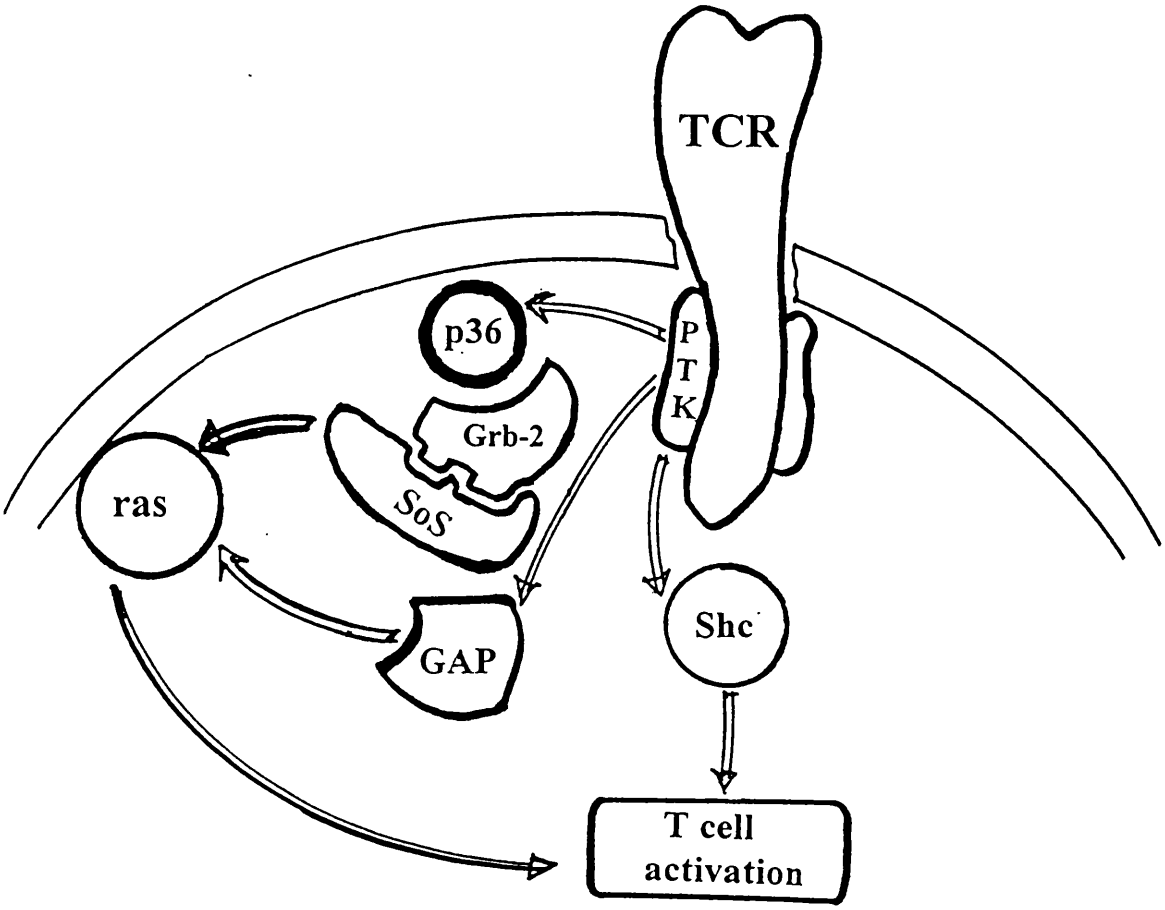
Stimulation of the *ras* activator, SOS is thought to be mediated by an adaptor protein, Grb-2, which recruits SOS to form a stable complex with *ras*. SH_2 and SH_3 domains (homologous to src kinases) are thought to be important in the interaction between Grb-2 and SOS (McMormick, 1993).

SH_2 and SH_3 domains are present in a large number of proteins involved in signal transduction. Consequently an SH domain within a single enzyme could interact with many different activators. Thus *ras* may serve as a multiple recipient to a variety of tyrosine kinases. To date, there are two proteins identified which have the potential to bind to Grb-2 SH_2 domains; a membrane-located 36kDa (p36) tyrosine phosphoprotein and two isoforms of an adaptor protein, Shc (Izquierdo *et al.*, 1995). It is proposed that when the TCR is activated, Shc and the membrane bound p36 are tyrosine phosphorylated and bind to Grb-2 SH_2 domains. The interaction between Shc and the TCRζ is transient, allowing Shc only to be tyrosine phosphorylated, preventing the formation of a stable TCR-Shc complex (see Figure 1.5).

Figure 1.5

Proposed summary of the events following TCR ligation.

Adapted from Izquierdo *et al.*, 1995.



Key.

SOS "son of sevenless"-like protein

GAP GTPase activating protein

PTK Protein tyrosine kinase

TCR T cell receptor

Following tyrosine phosphorylation, Shc dissociates from the receptor, forming a complex with the cytoplasmic adaptor molecules. p36 is thought to be responsible for linking the TCR-activated PTKs to Grb-2-SOS. In contrast, IL-2 receptor-activated cells show no tyrosine phosphorylation of p36 but the formation of Shc-Grb-2- SOS complexes. Consequently p36, could be a substrate for ZAP-70, which is not activated by the IL-2 receptor (Cantrell *et al.,* 1994). The exact role of p36 remains undetermined.

Interestingly, another *ras* feature is the failure of CD28 to regulate the guanine nucleotide binding cycle. This correlates with the inability of CD28 to induce tyrosine phosphorylation of p36 or Shc (Nunes *et al.,* 1994).

B. Signalling from *ras* to the nucleus.

The details of the intracellular signalling routes which link plasma membrane-associated *ras* to the nucleus are incomplete. Nevertheless, it is well documented that although *ras* function in T cell activation is essential, it is not sufficient for a full activation response (Cantrell *et al.,* 1994).

The transmission of signals from *ras* to the nucleus is regulated by the activity of MAP (mitogen activated protein) kinases. There are at least two MAP kinases in T cells: (extracellular signal regulated kinases) ERK1 and ERK2 (Leevers *et al.,* 1992). The activity of MAP kinase is controlled by an activator kinase, MAP kinase kinase (MAPKK). *Ras* and MAPKK are linked by at least one other kinase MAPKKK, the best characterised being Raf-1 (Avruch *et al.,* 1994). ERK2 activity is regulated by two co-existing pathways; one mediated by *ras* and one controlled by PKC. Although the TCR stimulates both, it is *ras* and not PKC which couples the TCR to ERK2. It is speculated that Raf-1 may provide the missing link between *ras*/PKC and ERK2 (Cantrell, 1994). (See Figure 1.6).

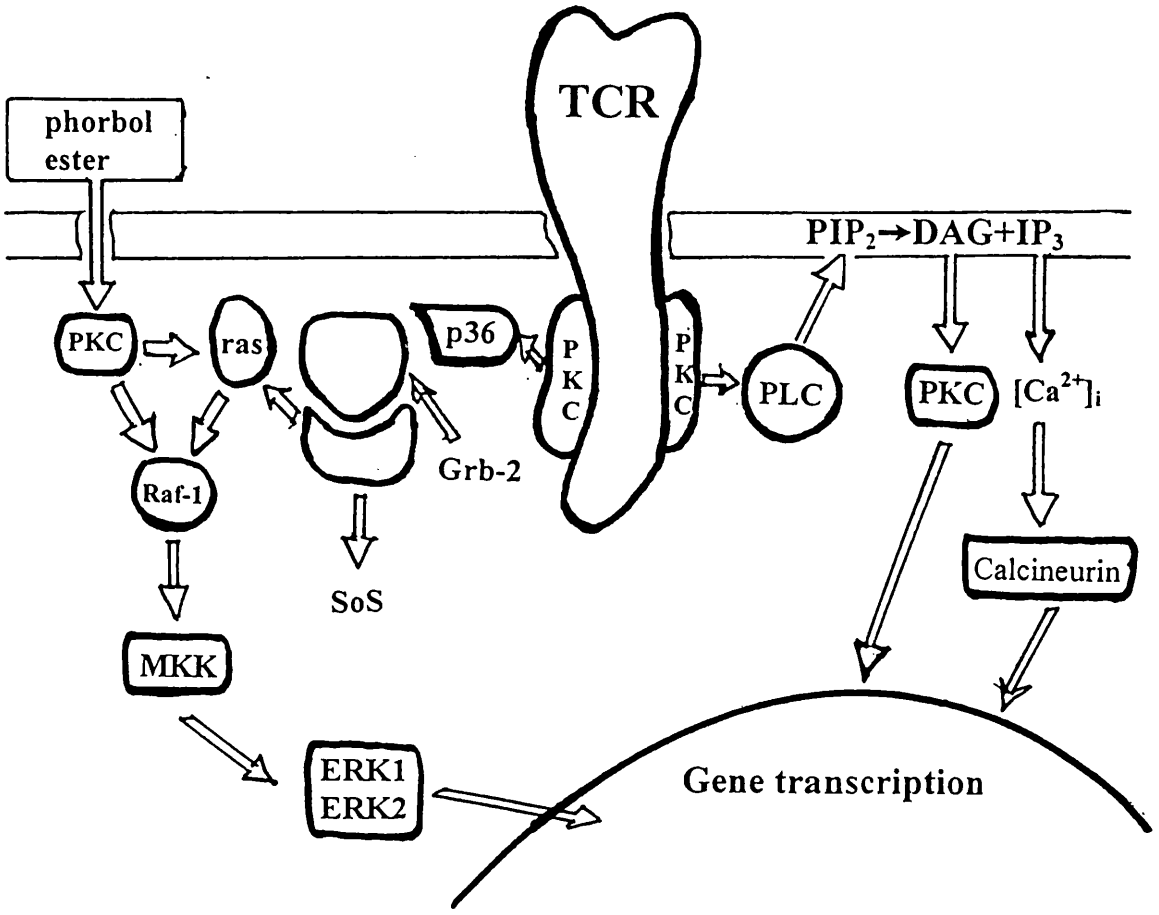
In addition, *ras* has been implicated as a target for free radicals and as a sensor of cellular redox status (Lander *et al.,* 1995). In support of a redox-mediated regulation of *ras,* a structural interaction between the nitric oxide radical and *ras* has been demonstrated (Lander *et al.,* 1996).

It is surprising that although the TCR and IL-2 receptor have distinct patterns of gene expression, they can regulate a common signalling molecule, *ras.* Thus the importance of this signalling molecule could be more complicated than outlined here, with the existence of many *ras* effectors, *ras*-Raf-1-MAPkinase being only one of them.

Figure 1.6

Regulation of *ras.*

Adapted from Izquierdo *et al.*, 1995.



Key.

PTK	Protein tyrosine kinase
$[Ca]_i$	intracellular calcium
MKK	mitogen-activated protein kinase kinase
ERK	extracellular-signal-regulated kinase

(For all other abbreviations see Figure 1.3)

1.2.5. IL-2 and the IL-2 receptor.

Interaction of IL-2 with the IL-2 receptor is vital for T cells to undergo G_1 progression, S phase transition (time of DNA replication) and subsequent cell division (Smith, 1988). Resting T cells neither produce nor respond to IL-2. A series of TCR specific and non-specific signals induce IL-2 gene expression and its regulation is a vital point in subsequent T cell activation. The events following IL-2 receptor stimulation leading upto cellular proliferation remain undefined. Nevertheless, many well-characterised biochemical pathways have been implicated. These involve tyrosine kinases, *ras* proteins and PI-3 kinase.

A. IL-2.

Human IL-2 is a 15kDa glycoprotein, containing one disulphide bridge essential for its biological activity. Production of IL-2 is confined primarily, but not exclusively, to CD4+ T lymphocytes (Kroemer and Wick, 1989). Expression and secretion of IL-2 requires the interaction of the TCR/CD3 complex with its specific antigen together with a secondary signal provided by accessory cells. The exact contribution provided by accessory receptor molecules such as CD2 and CD28 remains unclear but it has been shown that the signals from the TCR/CD3 complex can synergise with them to induce IL-2 gene expression. Since the requirements for T cell activation closely match those of IL-2 gene expression, IL-2 release is often used as a model for the study of signal transduction (Riegel *et al.*, 1992).

The effects of IL-2 have been well-documented. Its biological activities range from control of lymphocyte proliferation and differentiation to enhancement of immune and cell-mediated cytolysis of tumours and virus infected cells (Reed *et al.*, 1993). Consequently, IL-2 is thought to be a principal regulator of in vivo immune responses. All of the effects of IL-2 are mediated through binding to its specific membrane receptor.

B. IL-2 receptor.

The IL-2 receptor (IL-2 R) is a heterotrimeric complex of cell surface glycoproteins. It is composed of three distinct chains (α: 55kDa, β: 75kDa, γ: 64kDa), which bind IL-2 to generate a proliferative response (Nelson *et al.*, 1994). The three proteins are encoded by distinct and structurally unrelated genes.

IL-2 R are present on a wide variety of immunological cells. The IL-2 R β chain is expressed on quiescent macrophages and monocytes (Espinoza-Delgado *et al.*, 1990) and on mature CD4+ and CD8+ T cells (Nishi *et al.*, 1988). Resting T cells do not express IL-2 R α chains (Tac, CD25) but these are rapidly induced following TCR/CD3 activation. Although both IL-2 and IL-2 R α chains have been shown to be expressed following similar stimuli, expression of the receptor gene is not as stringently regulated as the IL-2 gene. PMA can activate the IL-2 R gene but IL-2 expression requires activation from both a calcium ionophore and PMA, demonstrating similar but distinct mechanisms of activation for both genes (Minami *et al.*, 1992).

In contrast to the IL-2 R α chain, the cytoplasmic region of the IL-2 R β chain is far larger, but contains no catalytic kinase motifs. By itself the IL-2 R α chain has a low affinity for IL-2 (10^{-8} M); insufficient for biological activity. The γ chain together with the β chain forms an intermediate affinity binding site for IL-2 which is capable of mediating the actions of IL-2 (Gaulton *et al.*, 1994).

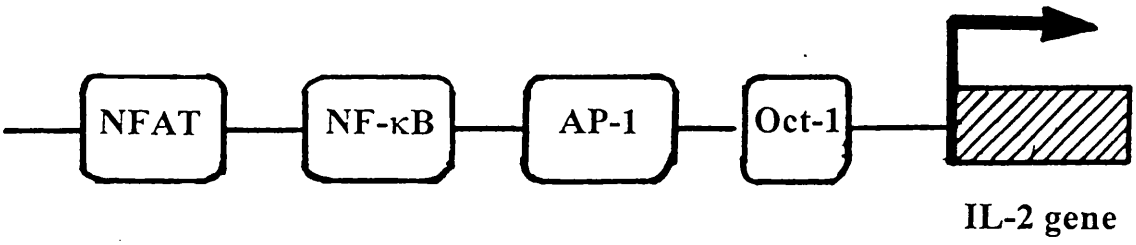
C. IL-2 and IL-2 receptor gene regulation.

The transcription of genes is influenced by various regulatory elements. These elements, referred to as promotors, enhancers and silencers, are composed of discrete DNA sequence motifs which form binding sites for specific binding proteins. Activation of the enhancer of the IL-2 and IL-2 R α chain genes requires the co-operative interaction of multiple transcription factors, each of which follows a different signalling pathway. Furthermore, activation of the IL-2 enhancer requires full occupancy, activation of only one of them cannot lead to IL-2 transcription.

At least four sequence motifs to which functionally relevant nuclear proteins bind have been identified in the 5' region of the gene encoding IL-2 (Ullman *et al.*, 1990). These include an NFAT-1 binding site, a NF-κB-like motif, two AP-1-like binding sites and a site for NF-IL-2A (see Figure 1.7). Two of these enhancers serve as TCR responsive elements, one of which is bound by a factor specifically induced in activated T cells (NFAT-1), the other by the constitutively produced factor NF-IL-2A, an oct-1 like factor that probably suppresses IL-2 transcription in resting T cells and must be modified or must cooperate with other binding factors to allow activity of the IL-2 promotor (Kroemer *et al.*, 1991).

Figure 1.7

The IL-2 promotor.



The promotor region of the IL-2 R α chain gene consists of a minimum of five positive regulatory elements and at least one negative element. Like the IL-2 gene, the enhancer region of the gene encoding IL-2 R α chain also contains an NF-κB binding site. The fact that the IL-2 and IL-2 R α chain genes share at least one regulatory element may explain why the two genes are often coexpressed (Hoyos *et al.*, 1989). In contrast to the IL-2 gene, which requires triggers from both the TCR and an accessory signal, the IL-2 R α chain gene can be expressed after activation with IL-1, PMA or TCR binding alone, without the apparent need for a second signal. Furthermore, IL-2 can induce the transcription of its own receptor (Leonard *et al.*, 1985).

NFAT, an inducible transcription factor, requires protein synthesis for its function. Signals derived from the TCR/CD3 complex or CD2 can synergise with both PKC

activators and calcium ionophores in the induction of NFAT expression. Thus, maximal NFAT expression requires a minimum of two signals for the synthesis of the nuclear targeted component of NFAT. Increases in calcium activate calcineurin, promoting the translocation of pre-existing cytosolic NFAT across the nuclear membrane, where it combines with its binding site (Lewis and Cahalan, 1995).

The functional activity of NF-κB in contrast, does not require protein synthesis but is modulated by an inhibitor protein, IκB. In its inactive form NF-κB is complexed to IκB within the cytoplasm. Upon activation I-κB is released and active NF-κB translocates to the nucleus where it interacts with its binding site (Edwards and Crabtree, 1989). The exact mechanism of IκB inactivation is controversial but occurs in the absence of protein synthesis. A variety of agents are recognised to be able to activate NF-κB, including IL-1 and PKC. However IL-2 mediated NF-κB activation is independent of PKC, suggesting an alternative non-PKC pathway of activation (Ghosh *et al.,* 1990). It is speculated that all these agents activate NF-κB through the production of ROS (Schreck, 1991). Recently the importance of calcineurin in NF-κB activation has been emphasised, which can act in synergy with PMA to activate NF-κB (Frantz *et al.,* 1994).

AP-1 is formed from a complex of c-fos together with c-jun. In T cells, members of the AP-1 family are essential for the formation of NFAT. Jun/fos dimers have been implicated in the molecular basis of T cell anergy (unresponsiveness); a likely mechanism for self tissue protection from immune responses mediated by tissue specific self antigens. Another interesting feature of AP-1 is that it can be activated by multiple signal transduction pathways, including phorbol esters. AP-1 recognises a conserved sequence which functions as a PMA (phorbol ester) responsive element. Furthermore, like NF-κB the activation of AP-1 appears to be mediated by redox processes (Devary *et al.,* 1991). Redox regulation will be discussed further in section 1.4.

D. IL-2 receptor signal transduction.

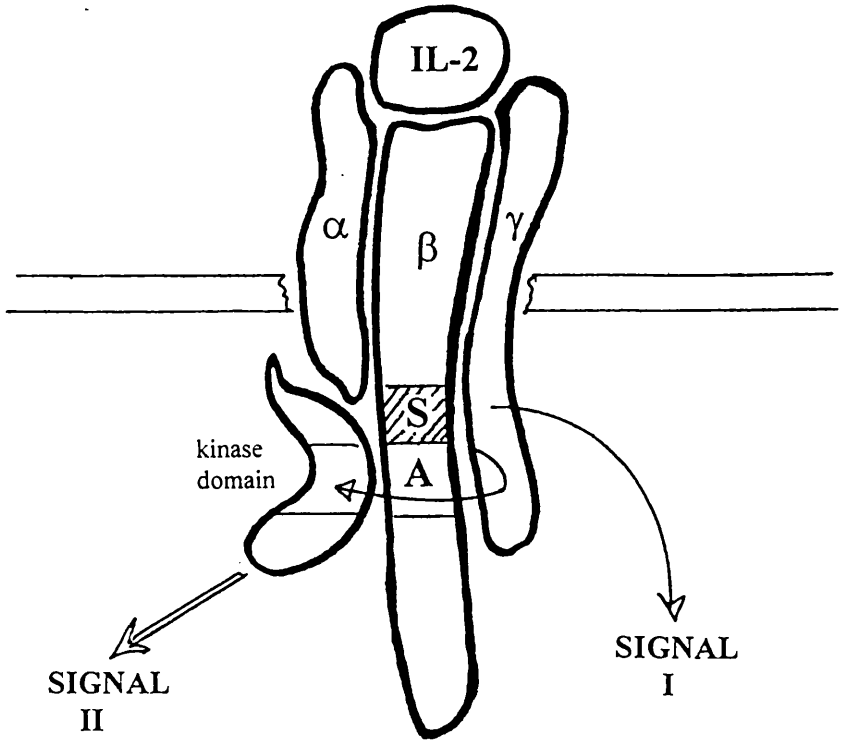
It is now well established that the IL-2 R is coupled to its own unique signalling pathway which complements TCR signalling, rather than duplicates it. One major difference is that unlike the TCR which regulates intracellular calcium, the IL-2 R does not (Mills *et al.*, 1985). Consequently calcium ionophores and phorbol esters can not substitute for IL-2 in the regulation of T cell proliferation (Cantrell and Smith, 1984). Furthermore, the tyrosine kinase cascade regulated by the two receptors is different. However, it is surprising that some signalling molecules are targets for both the TCR and IL-2 R. These include the PI-3 kinase and *ras*. The roles of these molecules will be discussed below.

(i) Tyrosine kinases.

The subunits of the IL-2 R have no intrinsic PTK activity, but like the TCR are thought to interact with and stimulate non-receptor kinases. Since the pattern of substrate phosphorylation seen after activation of the IL-2 R and TCR are different, it is unlikely that they regulate a common tyrosine kinase pathway (Taga and Kishimoto, 1993). IL-2 R β chain itself undergoes phosphorylation and is believed to be a direct substrate for the IL-2 R associated PTK (Nelson *et al.*, 1994; Fung *et al.*, 1991). Much controversy exists over the nature of IL-2 R associated protein kinases. Some studies have suggested that multiple PTK can associate with and are activated by IL-2 binding (Gaulton and Williamson, 1994), whilst others have proposed that the tyrosine kinase lck is physically associated with the IL-2 R β chain (Hatakeyama *et al.*, 1991). This latter proposal has been extended further by Minami *et al* (1992), who proposed that there are two different signals generated from the IL-2 R β chain which exert discrete signalling effects, with a possibility of convergence. See Figure 1.8.

Figure 1.8

Proposed model for IL-2 receptor-mediated signalling.



Key.

S "serine-rich"

A "acidic" region

SIGNAL I requires the "serine rich" region of the Il-2 R β chain and is essential for cell cycle progression.

SIGNAL II requires the "acidic" region of the β chain and is thought to be important for lck association.

Whilst it is well documented that the expression of CD45 is essential for TCR function and PTK regulation, IL-2 R signal transduction is independent of this tyrosine phosphatase (Cantrell *et al.*, 1993).

(ii) PI-3- kinase.

As mentioned previously, PI-3 kinase phosphorylates phosphoinositides at the 3 position of the inositol ring, generating a unique subclass of polyphosphoinositol lipids. It has been reported that both IL-2 R and TCR occupancy results in the rapid activation of PI-3 kinase (Merida *et al.*, 1991; Cantrell *et al.*, 1993). Subsequently tyrosine phosphorylation of p85 (regulatory subunit of PI-3 kinase) has been shown to be one of the earliest detectable markers of IL-2-dependent PTK activation. However, the mechanism whereby this common signalling target generates a response remains to be established.

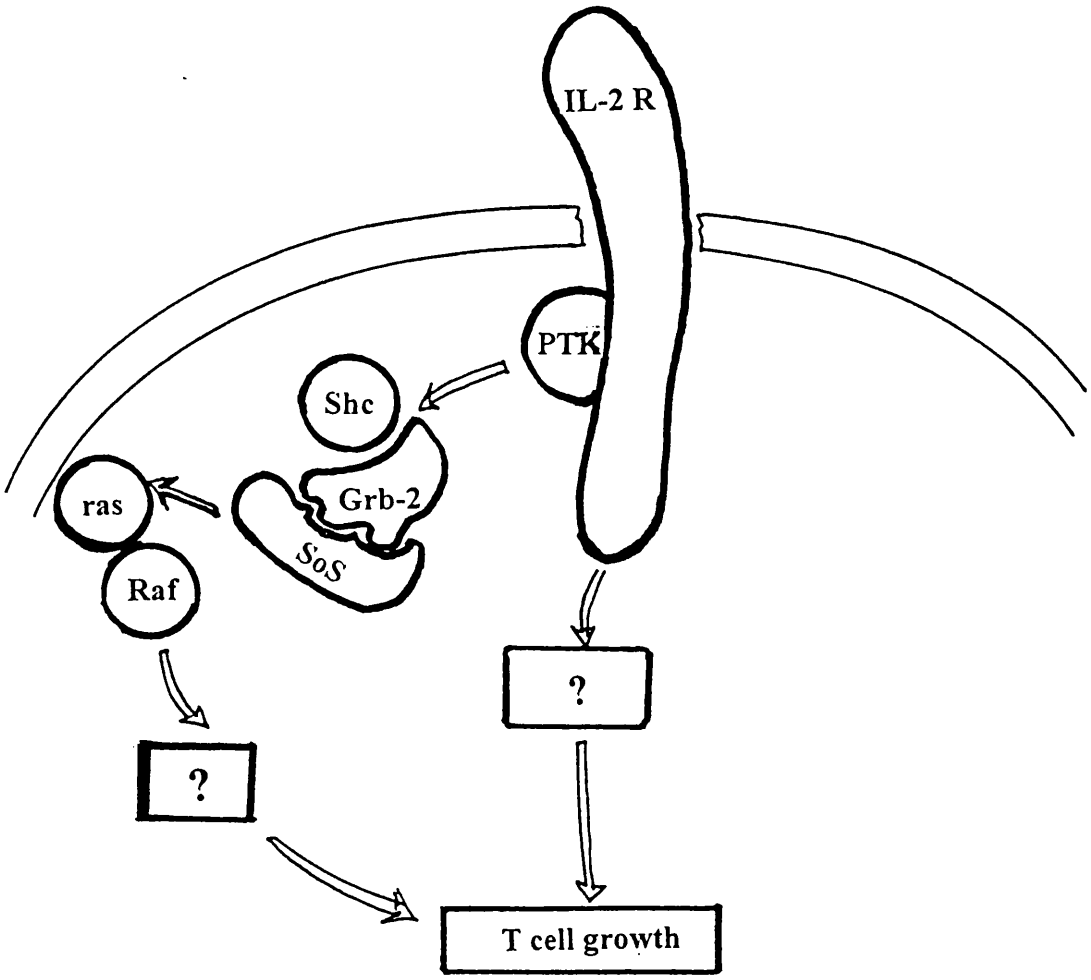
(iii) Ras.

Recent studies have shown that IL-2 stimulation increases the formation of active *ras* (Graves *et al.*, 1992; Rayter *et al.*, 1992; Izquierdo *et al.*, 1993). *Ras* regulation of the IL-2 gene is thought to be mediated by NFAT, and it has been demonstrated that *ras* and calcineurin can synergise for NFAT induction (Woodrow *et al.*, 1993). In contrast to the TCR, IL-2 R activation stimulates Raf-1, but there is much debate about whether IL-2-mediated ERK 2 stimulation occurs (Pastor *et al.*, 1995). The current hypothesis for IL-2 R *ras* regulation is that it regulates SOS via the adaptors Shc and Grb-2 (Cantrell *et al.*, 1994). Although the mechanisms of *ras* activation in the two systems are known to be distinct, many details remain to be determined. See Figure 1.9.

Figure 1.9

Proposed model for *ras* regulation by the IL-2 receptor.

Adapted from Izquierdo *et al.*, 1995.



Key

IL-2 R IL-2 receptor

PTK Protein tyrosine kinase

SOS "son of sevenless"-like protein

? novel protein

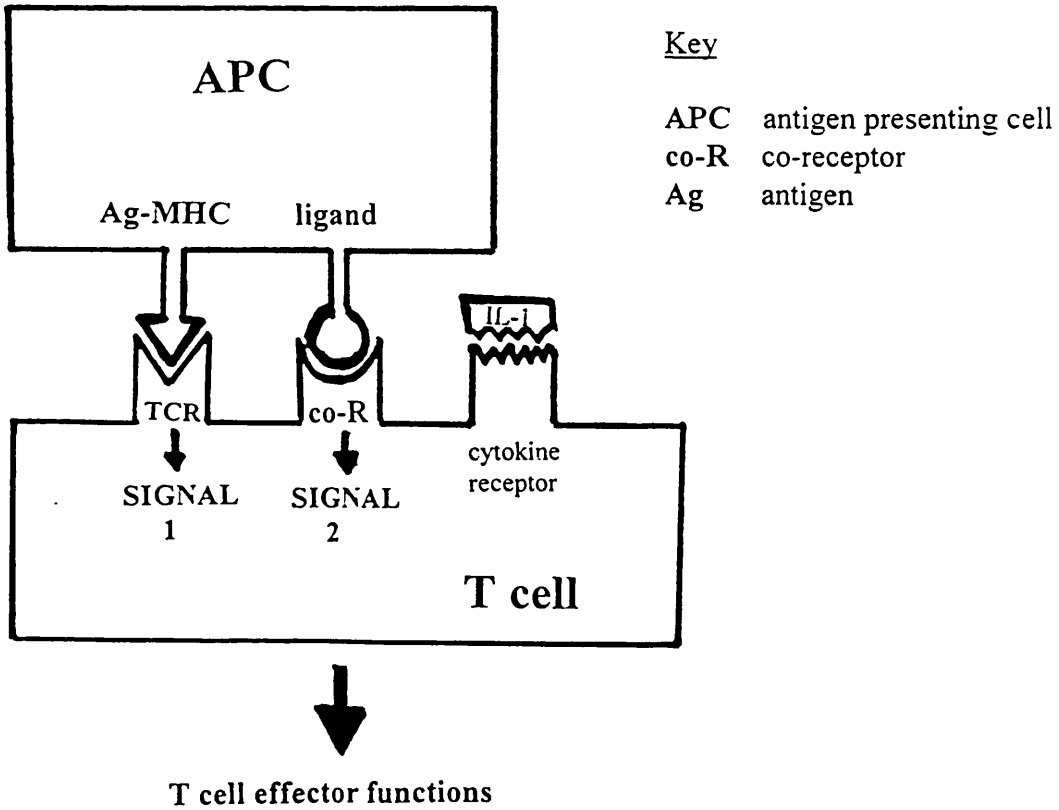
1.2.6. Role of accessory molecules.

Contact between the TCR and the antigen/MHC is by itself insufficient to stimulate resting cells to pass through G_1 into S phase of the cell cycle. A critical factor in determining entry into the S phase is the amount of growth factor produced, in particular IL-2 as discussed above. To initiate sufficient production of IL-2 there is a requirement for the so-called "accessory" signal. In T cells, this second signal is mostly derived from contact with an APC. This idea is supported by the observation that purified T cells do not produce IL-2 in response to mitogenic lectins or anti TCR antibodies in the absence of accessory cells, despite the fact that TCR-associated second messengers are generated (Jenkins and Johnson, 1993).

A two signal hypothesis for activation was first proposed by Lafferty and co-workers (1970) [see Figure 1.10]. Signal 2 can be delivered either in the form of a soluble mediator (IL-1) or by contact with an APC.

Figure 1.10

Model of two signal hypothesis for T cell activation.



Many T cell surface molecules have been recognised which can act in concert with TCR-mediated activation (signal 1) to allow full activation. Potential signal 2 T cell receptors can be: (i) adhesion receptors, promoting intercellular adhesion between the T cell and the APC thus indirectly enhancing/prolonging TCR coupled signal transduction, or (ii) receptors which generate a co-stimulatory signal independent and biochemically distinct from those generated by the TCR. However, this criterion is not always so simple since cross-linked antibodies can in some cases augment signal 1, eg anti-CD28. In addition, some receptors can serve more than one function, eg. CD2 has potent roles in adhesion and in the delivery of a co-stimulatory signal.
The list of cell surface molecules involved in T cell activation is vast but only the role of CD28 and CD2 will be discussed here.

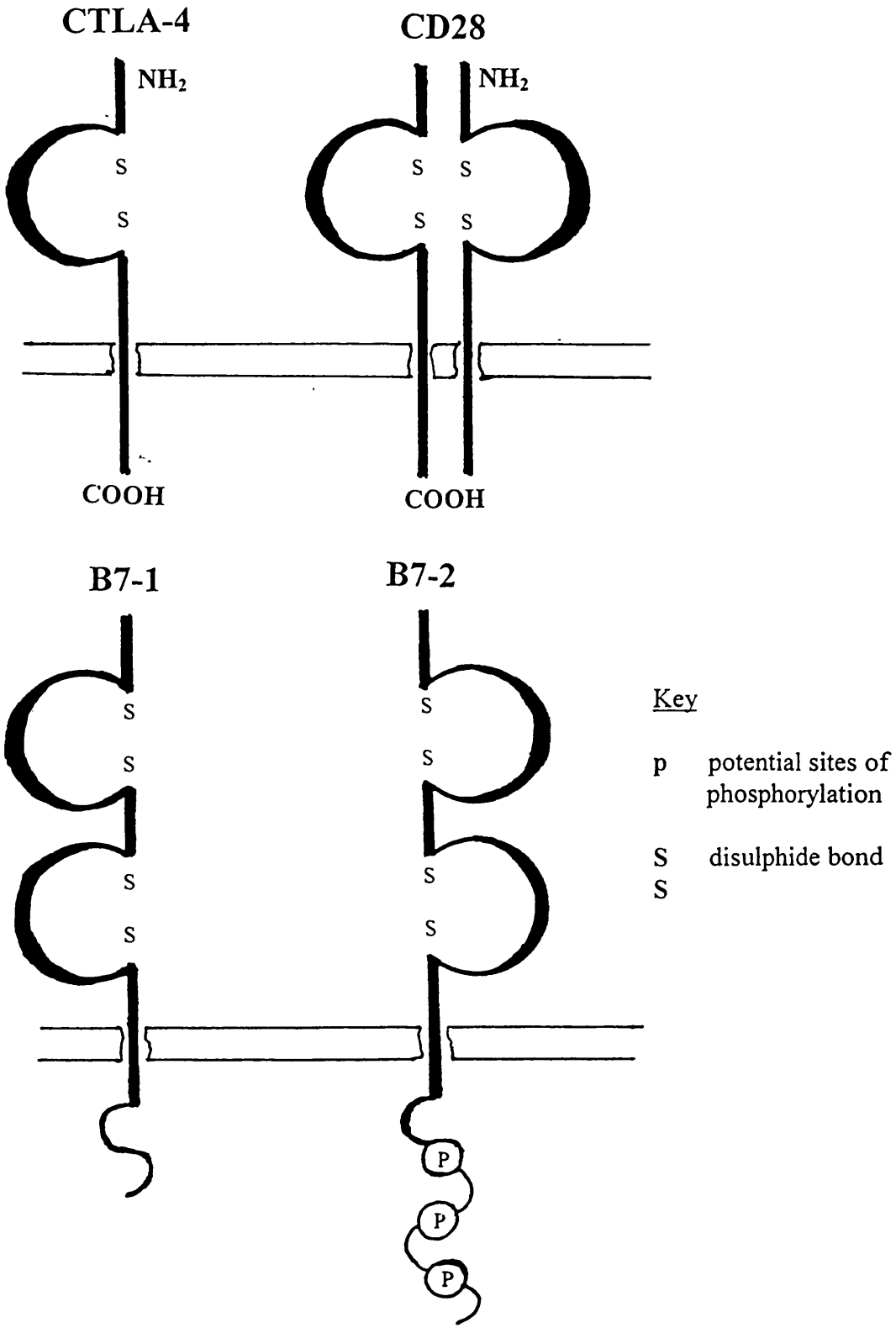
A. CD28.

To date there are two members of the CD28 gene family: CD28 and CTLA-4. CD28 is a cell surface glycoprotein, composed of two identical 44kDa subunits. Human peripheral blood T cells are 80% CD28+ (June *et al.*, 1994). In contrast to CD28, CTLA-4 (cytolytic associated antigen) is expressed on T cells only after activation. Despite the similarities between the CD28 and CTLA-4 cytosolic domains, the functional role of CTLA-4 remains unclear. A recent study has suggested that CTLA-4 associated with a tyrosine phosphatase may be involved in down-regulation of T cell activation (Marengere *et al.*, 1996). The exact implications of these findings will not be discussed here.
CD28 binds to at least two ligands on APCs, B7-1 and B7-2 (also known as B cell activation antigens B7/CD80 and B70/CD86 respectively) [See Figure 1.11]. B7 can be expressed on a variety of cell types including dendritic cells, activated macrophages, B cells and T cells (June *et al.*, 1994).

Figure 1.11

The B-7 and CD28 receptor families.

Adapted from June *et al.*, 1994.



Many different monoclonal antibodies to CD28 are available, eg. 9.3, Kolt2, 15E8. Most of these antibodies bind to the site of the natural ligand although some bind to distinct epitopes. Accordingly it has been shown that different CD28 monoclonal antibodies with distinct binding properties differ in their ability to induce T cell activation (Nunes *et al.*, 1993).

Ligation of CD28 alone has little effect on T cell activation, but can act in synergy with a range of "signal 1" stimuli to induce activation. Accumulating evidence suggests that CD28 provides signals distinct from those generated through the TCR/CD3 complex. CD28 has been shown to synergise with PMA, calcium ionophores, CD2 and CD3 to induce IL-2 production and proliferation of purified T cells (Ledbetter *et al.*, 1990; Costello *et al.*, 1993).

It is well established that low concentrations of CD28 and PMA can induce IL-2 mRNA, via CD28-mediated stabilisation of processed cytoplasmic mRNA (Lindsten *et al.*, 1989). In addition it has been shown that CD28, in combination with PMA, can also induce TNFα, interferon γ and GM-CSF (Thompson *et al.*, 1989). If T cells are stimulated with CD28 for long periods of time (>6 hours), increases in the actual transcription of IL-2 mRNA aswell as mRNA stabilisation occurs. This later CD28 action is distinct to its early action. In contrast to its early action, the later one generates increases in intracellular calcium, IP_3 production and is cyclosporin A (CsA)-sensitive. CsA is a tool often used to distinguish calcium-associated signals, since it inhibits responses to calcium mobilisation through its effects on calcineurin (Gelfand *et al.*, 1987).

Studies with the IL-2 promotor have recently identified a CD28-responsive element (CD28RE) which binds NF-κB-like members, following stimulation with PMA plus anti-CD28, or anti-TCR plus anti-CD28 (Ghosh *et al.*, 1993; June *et al* 1994). Subsequently, NF-κB activation (Baeuerle *et al.*, 1994) and CD28-mediated increase in mRNA transcription (Los *et al.*, 1995a) have been linked with ROS production.

Much controversy exists over the nature of the biochemical signals delivered early after CD28 crosslinking. CD28 increases tyrosine phosphorylation of cellular substrates distinct from those induced by the TCR (Lu *et al.*, 1992). Indeed, it does

not affect tyrosine phosphorylation of the TCRζ chain, implying that it does not activate ZAP-70. This pathway of activation is CsA-resistant but the exact nature of the tyrosine kinases and phosphatases mediating CD28 signalling are unclear. At later time-points, CD28 cross-linking induces tyrosine phosphorylation of PLCγ1 and recruits lck and fyn amplifying TCR-mediated responses. This pathway is CsA-sensitive. In contrast to TCR/CD3 responses, which can be completely blocked by CsA, CD28-mediated responses are often only partly inhibited by CsA (Williams *et al.*, 1992). Consequently, it is thought that the CD28 receptor can deliver at least two distinct signals, dependent on the time of receptor cross-linking.

Of greatest interest is the recent finding that CD28/B7-1 signalling can increase cellular tyrosine phosphorylation and activation of PI-3-kinase directly (Ward, 1996). The cytoplasmic domain of CD28 contains a specific motif which is a binding site for the SH_2 domain of PI-3-kinase (August *et al.*, 1994; Stein *et al.*, 1994). Thus the phosphorylation of CD28 and its subsequent binding to PI-3-kinase may be an important event in the co-stimulation signalling of CD28. This PI-3-kinase-mediated pathway is also CsA sensitive. It is speculated that PKCζ, an atypical PKC isoenzyme which is insensitive to PKC, is activated by PI-3 kinase and this may explain the observed synergy between CD28 and PMA (See Figure 1.12).

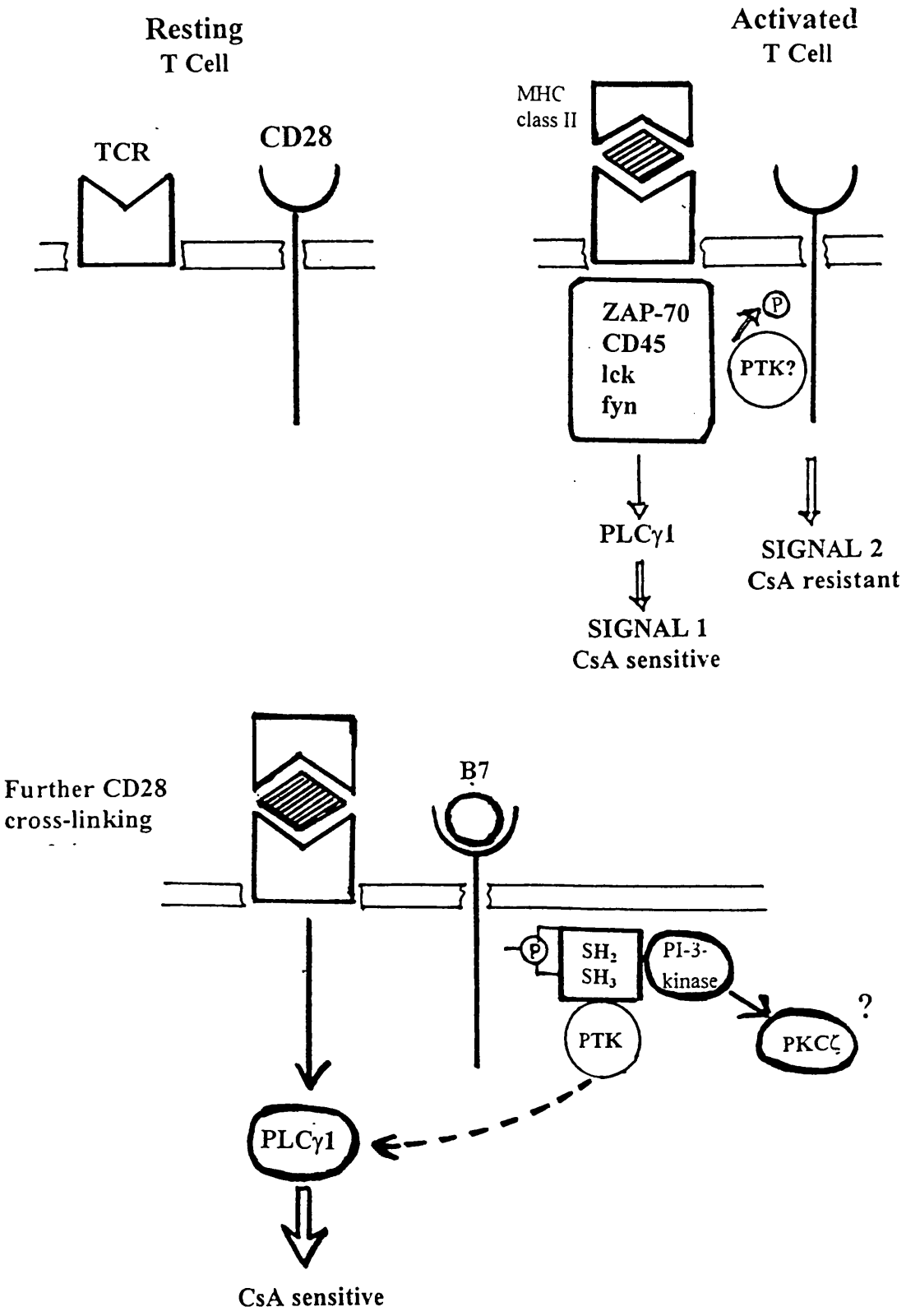
Much conflicting evidence exists over the role of *ras* in CD28-mediated signalling. Whilst some studies suggest that CD28 does not activate *ras* or induce the tyrosine phosphorylation of Shc or any other protein capable of recruiting Grb-2-SOS complex to the membrane (Nunes *et al.*, 1994), others suggest it can bind to the complex Grb-2-SOS via its SH_2 domain (Raab *et al.*, 1995). The latter study also suggested that CD28 can bind to a T cell specific src-like protein tyrosine kinase, itk. However the exact implications of these findings remain to be established.

The underlying end-point of CD28/B7 action is unclear. A possible explanation may come from its ability to elevate c-jun mRNA. Decreased binding of the jun/fos dimers to the IL-2 promotor have been implicated in the molecular basis of T cell anergy (unresponsiveness). Indeed CD28/B7 interactions have been shown to prevent and reverse T cell anergy (Romano *et al.*, 1993).

Figure 1.12

Proposed model of signal transduction mediated by the CD28 receptor.

Adapted from June *et al.*, 1994.



B. CD2.

The CD2 cell surface receptor is a 45-50 kDa glycoprotein, expressed on virtually all human T cells. Human CD2 mediates the rosetting capacity of peripheral T lymphocytes to sheep erythrocytes receptors, a process classically used to isolate human T cells from the mononuclear population (Turco *et al.*, 1988).

CD2 was the first T cell molecule identified for which dual roles in adhesion and activation existed. CD2 molecules are ideally situated to transduce information to the cell once contact has been made, and also provide a co-stimulatory signal.

It is now well established that the ligand for CD2 is the structurally related molecule CD58 (LFA-3, leukocyte function associated-3 molecule). CD58 is a phosphatidylinositol anchored, transmembrane 55-70 kDa glycoprotein, with a broad tissue distribution. In addition, it has recently been proposed that CD59 on the surface of erythrocytes may be a second ligand for CD2 (Deckert *et al.*, 1995).

An intriguing feature of CD2 is that it can function as an activator or inhibitor in T cell activation, depending on the combination of anti-CD2 antibodies used. Three different epitopes have been mapped to three distinct regions of the extracellular segment of the CD2 molecule. These epitopes can be either external (high surface density) or internal (low surface density). Internal epitopes are increased following activation or monoclonal antibody binding to an external epitope. Accordingly, CD2 has three distinct epitopes termed $T11_1$, $T11_2$ and $T11_3$.

$T11_1$ and $T11_2$ are expressed on resting and activated T cells. $T11_3$ is thought to be restricted to activated T cells, although its presence on resting cells is still controversial (Kabelitz, 1990). $T11_1$ mediates the phenomenon of rosette formation and monoclonal antibodies directed against $T11_1$ (known as the E-receptor) inhibit rosette formation and certain other T cell responses (Peterson and Seed, 1987). It is speculated that the structure detected by the $T11_1$ functions as a "negative signal receptor". Paradoxically, the combined action of monoclonal antibodies directed against appropriate combinations of external and internal CD2 epitopes can be mitogenic for resting T cells and T cell clones in the absence of accessory cells. This

latter mode of activation is referred to as the "alternative pathway" (Alcover *et al.*, 1988). The nature of the biochemical messengers mediating these two contrasting effects are incomplete. Recent developments in the nature of the biochemical second messengers generated by signalling through $T11_1$ will be discussed first, followed by those mediating the "alternative pathway of activation".

External epitopes recognised by monoclonal antibodies that block E rosetting (eg. 9.6, OKT11) have been shown to suppress mitogen-induced T cell proliferation. Supporting the existence of a negative signalling pathway, anti-CD2 monoclonal antibodies have been shown to completely block antigen-induced IL-2 production (Ohno *et al.*, 1991). However $T11_1$ cannot inhibit IL-2-dependent proliferation of T cells already expressing IL-2, suggesting that it exerts its action before T cells acquire sensitivity to IL-2 (Palacios *et al.*, 1982). Furthermore, the efficacy of antibodies to $T11_1$ to inhibit T cell proliferation is dependent upon the type of stimulant used. They only partly inhibit responses by plant lectins, completely suppress proliferation induced by calcium ionophores but not phorbol esters. Thus, it is speculated that the receptor recognised by $T11_1$,known as the E receptor, may influence the mechanism by which antigens and mitogens increase intracellular calcium (Palacios and Martinez-Maza, 1982).

A second messenger responsible for the negative regulation of T cell responses is cAMP (Kammer, 1988). It could be that $T11_1$ suppresses lymphocyte responses by increasing intracellular cAMP levels. However, much conflicting data exists over a role for cAMP as a mediator in $T11_1$ inhibition. Whereas some studies show that CD2/LFA-3 binding increases cAMP in human T cells (Carrera *et al.*, 1988) and that agents which elevate cAMP can upregulate CD2 (Collins *et al.*, 1994), others have failed to see any increase in cAMP levels upon stimulation (Ohno *et al.*, 1991).

CD2 has been found to be physically associated with CD45 on the surface of T cells (Schraven *et al.*, 1990). Furthermore, CD45-like monoclonal antibodies can selectively modulate T cell activation through CD2 (Ledbetter *et al.*, 1988). Whether CD2 alters the phosphatase activity of CD45, resulting in negative regulation of the

initial activation signal, and whether this action is dependent on the TCR complex, remains unclear.

Whilst individual anti-T11 monoclonal antibodies are unable to induce activation, combinations of anti-$T11_2$ plus anti-$T11_3$ (to a lesser extent certain anti-$T11_1$ plus anti-$T11_3$) can induce accessory cell-independent T cell activation, measured by proliferation and IL-2 production (Silciano *et al.*, 1985). However, $T11_1$- plus $T11_3$-induced T cell activation is IL-2-dependent (Meuer *et al.*, 1984). Activation via this alternative pathway of activation does not need cross-linking with other cell surface molecules and is distinct from that induced by the TCR (Weiss and Imboden, 1987).

However, several observations suggest an interdependence of the CD2 and TCR-CD3 activation pathways. For example, loss of surface CD3 blocks the T11 pathway of activation (Kabelitz, 1990). Paradoxically, modulation of T11 does not affect the cells ability to be triggered through the TCR-CD3 complex (Pantaleo *et al.*, 1987), possibly through a requirement of the CD3ζ sub-unit (Moingeon *et al.*, 1992).

Furthermore, much controversy exists over whether the synergism between anti-CD2 ($T11_3$) and anti-CD3 in inducing purified T cell proliferation is partly a result of a physical association between the two molecules (Bretmeyer *et al.*, 1987). Although it is well documented that an immediate consequence of T cell activation via the TCR/CD3 complex and CD2 antigen is the hydrolysis of PIP_2 and generation of IP_3 and DAG (Kanner *et al.*, 1992), it has recently been shown that they can both generate lipid products of PI-3-kinase (Ward *et al.*, 1992). This additional similarity in the early signal transduction events mediated by TCR/CD3 and CD2 pathway could mean that they are one and the same. Hence, seemingly conflicting data exist with respect to a possible requirement for a functional TCR-CD3 complex in signalling via the alternative CD2 pathway. Clearly more work needs to be done to determine the point of convergence of CD2 and TCR-CD3 signal transduction pathways.

Like the TCR/CD3 specific pathway, activation by a combination of CD2 monoclonal antibodies increases intracellular calcium (Ledbetter *et al.*, 1988), which cannot be simply explained by the CD2 molecule acting as a calcium channel (Bierer *et al.*,

1989). The pattern of tyrosine phosphorylation following the binding of anti-CD2 antibody pairs is unique and the fact that some phosphorylation can be achieved in the absence of TCR/CD3 expression, suggests that CD2 uses protein tyrosine kinases distinct from those stimulated by the TCR/CD3 pathway (Hubert *et al.*, 1993). Thus CD2 signalling may be dependent on an unidentified protein tyrosine kinase. Recently it has been shown that CD2 can regulate *ras* activation. This low molecular weight G protein could be part of the signal transduction responses influenced by CD2 (Graves *et al.*, 1991).

Another role of CD2 mentioned earlier is its participation in antigen recognition by facilitating antigen independent cell-cell adhesion (Hahn *et al.*, 1993). The adhesive and co-stimulatory pathways mediated via CD2 are thought to be mediated by distinct mechanisms.

It is speculated that binding of CD2 to its ligand leads to close contact between multiple zones of the T cell and APC. On resting T cells the TCR/CD3 complex and other molecules involved in antigen recognition are randomly distributed amongst larger molecules, such as CD45. CD2 may exclude CD45 from the contact region thereby extending the time for completion of steps involved in the signal transduction pathway (Davis *et al.*, 1996).

Progress has been made in elucidating features of the many steps in the transduction pathway which has contributed to the understanding of the multiple "transduction molecules". How these "transduction molecules" permit distinct activation programmes remains to be established. It has been suggested that various signalling strategies may be used, some of which may be regulated by reactive oxygen species (ROS) . In the next section the nature of ROS will be discussed and how the excess production of free radicals can contribute to cellular damage.

1.3 Reactive oxygen species.

1.3.1. Definition of a free radical.

A free radical can be defined as a chemical species, capable of independent existence that contains one or more unpaired electrons (Gutteridge, 1994). An electron occupying an atomic or molecular orbital by itself makes it highly reactive. Both atomic oxygen (O) and molecular oxygen (O_2) contain unpaired electrons. Atomic oxygen contains one unpaired electron in its outer valence shell and molecular oxygen has an outer valence shell containing two electrons, which are not spin-paired but remain as two unpaired electrons.

Oxygen-derived reactive species include singlet oxygen, superoxide radical anion, hydrogen peroxide and the hydroxyl radical. Its important to note that despite hydrogen peroxide being a ROS, it is not a radical since all its outer valence shell electrons are paired.

1.3.2. Production and properties of ROS.

In biological systems, reduction of molecular oxygen to water via a series of one electron transfer reactions results in the formation of radicals or reactive intermediates.

$O_2 + e^- \rightarrow O_2^- \bullet$	Superoxide anion radical
$O_2^- + e^- + 2H^+ \rightarrow H_2O_2$	Hydrogen peroxide
$H_2O_2 + e^- \rightarrow OH^- + OH\bullet$	Hydroxyl radical
$OH\bullet + e^- + H^+ \rightarrow H_2O$	

Radical reactions are generally chain reactions and can be divided into three distinct phases; initiation, propagation and termination. It is important to note that the

outcome of radical reactions can be very extensive and below is an overview of the biologically relevant chain reactions.

In the initiation phases O_2, H_2O_2 and water are the major substrates. O_2 acts as an electron acceptor for the single electrons generated from either one electron enzymatic reduction of endogenous or organic molecules, or as side products of respiration in mitochondria, endoplasmic reticulum (ER) or nuclear membranes.

$$O_2 + e^- \rightarrow O_2^-$$

Intracellular radicals are likely to be formed by photolysis, bond homolysis or redox reactions.

a. Bond homolysis. Here a covalent bond splits generating two radicals. The main criterion for bond homolysis is the existence of a weak bond, which in uncatalysed circumstances is rare. Organic disulphides and organic hydroperoxides (in polyunsaturated fatty acids) are likely examples.

b. Photolysis. Light can initiate radical reactions due to molecules absorbing energy. For example H_2O_2 can absorb energy resulting in the formation of OH•.

c. Redox reactions. This involves a one electron oxidation or reduction of organic molecules and often results in the formation of the superoxide radical anion. Redox reactions can involve complexed or chelated transition metal ions (mainly iron, but also copper, chromium and vanadium), which can behave as either catalysts or enzyme binding sites. An example is the Haber-Weiss reaction where iron or copper catalyses the decomposition of H_2O_2 or an hydroperoxide to produce OH• or RO•.

$$\begin{array}{ccccccc} H_2O_2 & & Fe^{2+} & & OH\bullet & & Fe^{3+} \\ \text{or} & + & \text{or} & \rightarrow & \text{or} & + & \text{or} \\ \text{R-OOH} & & Cu^{2+} & & RO\bullet & & Cu^{2+} \end{array}$$

In the propagation phase the number of radicals remains unchanged. In biological systems this can occur by either:-

a. Atom (or group) transfer. This often involves the outermost atom on the molecule eg. hydrogen. Hydrogen atom abstraction reactions occur in membrane damage, whereby a hydrogen atom is abstracted from phosphatidylcholine forming water and another radical (unpaired electron is on the carbon atom). DNA and RNA mutations and strand breakages are a consequence of hydogen atom addition to purine and pyrimidine bases.

b. Electron transfer. As the term suggests, an electron is transferred from a radical or radical anion to a non-radical substrate generating another radical. For example lipid peroxidation involves a one electron transfer from transition metal ions to peroxides.

The termination phase refers to the removal of radicals and the termination of the chain reaction. This can occur by either electron transfer (eg. dismutation reactions) or radical scavenging. Scavenging reactions generate radicals which are less likely to propagate the reaction, and form the basis of the body's natural antioxidant system.

A. Singlet oxygen.

By definition this is not a free radical because it does not have an unpaired electron. The removal of the spin restriction (two unpaired electrons with parallel spins) makes it highly reactive (Halliwell and Gutteridge, 1989). In biological systems, singlet oxygen is primarily produced by photosensitization. Here, light excites an organic molecule, the excitation energy is transferred to an adjacent oxygen molecule converting it to a singlet state, ultimately returning to the ground state itself. These organic molecules are referred to as photosensitizers and include flavins, bile pigments and porphyrins. The pathology of the porphyrias has been attributed to a porphyrin accumulation in the skin resulting in excessive singlet oxygen production. Singlet oxygen has also been implicated in the development of cataracts in the lens of the eye (Halliwell and Gutteridge, 1989).

Eosinophils can be responsible for singlet oxygen production. In contrast to neutrophils (which contain iron-chlorine myeloperoxidases) eosinophils contain unique peroxidases which oxidise bromide to hypobromous acid.

$$H_2O_2 + Br^- \rightarrow HOBr + OH^-$$

In addition to HOBr being a powerful antimicrobial agent, it can react with hydrogen peroxide to generate singlet oxygen.

As an excited state of molecular oxygen, singlet oxygen tends spontaneously to inactivate to regenerate molecular oxygen. Consequently, this results in light emission or energy transfer, allowing another molecule to enter an excited state. This process is called quenching. Vitamin A and E are well-known singlet oxygen quenchers.

B. Superoxide radical anion ($O_2^{-}\bullet$).

The addition of a single electron to singlet oxygen results in the formation of the superoxide radical anion ($O_2^{-}\bullet$). The properties of $O_2^{-}\bullet$ are determined by the nature of its immediate environment ie. aqueous or organic. In organic (non polar) solutions it is more stable, acting as a strong reducing agent (electron donor).The major role $O_2^{-}\bullet$ plays in oxygen toxicity is as a reducing agent for metal ions eg. iron complexes in cytochrome C (Gutteridge, 1994).

$$Fe^{3+} + O_2^{-}\bullet \rightarrow Fe^{2+} + O_2$$

This predisposes the cell to the deleterious effects of the extremely reactive hydroxyl radical, via the Fenton reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- \div OH\bullet$$

In aqueous solutions $O_2^{-}\bullet$ behaves as a weak oxidising agent, oxidising molecules such as ascorbic acid and thiols.

Any biological system generating $O_2^{-}\bullet$ will ultimately produce hydrogen peroxide via dismutation. The reaction below is catalysed by the enzyme superoxide dismutase (SOD), previously known as erythrocuprein.

$$O_2^{-}\bullet + O_2^{-}\bullet + 2H^+ \rightarrow H_2O_2 + O_2$$

Two types of SOD have been identified in mammalian cells, the copper-zinc enzyme located in the cytosol and the manganese enzyme found mainly in the mitochondria. Both enzymes catalyse the dismutation reaction with the same efficiency.

Production of $O_2^{-}\bullet$ occurs primarily in two ways: as by-products of various enzyme-substrate reactions, for example in the xanthine-oxidase/xanthine-dehydrogenase-system and mitochondrial electron-transport systems. Xanthine oxidase was the first documented source of $O_2^{-}\bullet$ (McCord *et al.*,1968). It catalyses the oxidation of hypoxanthine to uric acid with the generation of $O_2^{-}\bullet$ and hydrogen peroxide. Under normal conditions, the predominant form of xanthine dehydrogenase does not, however, generate any ROS, as it uses NAD^+ rather than oxygen as an electron acceptor. It is speculated that during the reintroduction of oxygen in ischaemic tissue, proteolytic enzymes convert xanthine dehydrogenase into an oxidase form, whereupon it generates ROS. Studies have demonstrated the accumulation of hypoxanthine in ischaemic myocardial tissue, thus acting as a substrate for $O_2^{-}\bullet$ production (Das and Engelman, 1990). However, the exact toxicological relevance of $O_2^{-}\bullet$ in reoxygenation injury remains to be determined. Other reports have implicated ROS in the pathology of gastric mucosal injury during ischaemia/reperfusion theory (Roberfroid *et al.*, 1995).

Much evidence exists that particle engulfment by neutrophils and macrophages is accompanied by a burst of oxygen consumption (Winterbourn, 1990). This oxygen consumption has been shown not to be due to conventional respiration but involves stimulation of the hexose monophosphate shunt and glucose metabolism via a membrane-bound flavo-protein, "the respiratory burst oxidase" (Babior, 1988). This enzyme catalyses the one electron reduction of oxygen to $O_2^{-}\bullet$ at the expense of NADPH (Nicotinamide Adenine Diphosphate) which is oxidised to $NADP^+$.

$$O_2 + NADPH \rightarrow O_2^{-}\bullet + NADP^+ + H^+$$

$O_2^{-}\bullet$ generation forms the basis of an important sector of host defence and studies performed with phagocytes in anaerobic conditions have demonstrated incomplete killing of bacteria (Sies *et al.*,1991). Recognised components of the NADPH/NADH oxidase are cytochrome-b and a flavoprotein. Enzyme activation results in electrons being transferred from NADPH via the flavoprotein to cytochrome-b, which serves as an electron donor to dioxygen (Rossi, 1986). Much controversy still exists about the exact mechanism of the phagocyte NADPH oxidase system. It has now been proposed that B lymphocytes (Maly, 1990) and T lymphocytes (Pick *et al.*,1988) may possess a NADPH-like oxidase system.

Evidence to date indicates that $O_2^{-}\bullet$ has limited reactivity with lipids, carbohydrates or nucleic acids because it is unable to cross biological membranes (Halliwell and Gutteridge, 1989). In normal conditions, most of the produced $O_2^{-}\bullet$ is accurately directed against invading organisms. However, under conditions of chronic inflammation such as rheumatoid arthritis, surrounding tissues are exposed to high levels of $O_2^{-}\bullet$. Since blood is in intimate contact with all body tissues a potential source for oxidative damage is linked with the increased $O_2^{-}\bullet$ leakage from erythrocytes during hypoxia (Levy, 1988).

$$\text{Haemoglobin (Hb)}O_2 \rightarrow Hb^{+} + O_2^{-}\bullet$$

$O_2^{-}\bullet$ has also been implicated in the pathogenesis of skin carcinoma. In vitro studies have demonstrated a link between protein kinase C activation, production of $O_2^{-}\bullet$ and phorbol esters in the promotion of skin carcinogenesis. In support of this hypothesis SOD has been beneficial in treating the condition (Roberfroid *et al.*, 1995). Paradoxically, $O_2^{-}\bullet$ has recently been identified in human amnion cells having a possible role in intrauterine contractions during labour (Masomoto *et al.*, 1990). Another study has shown that $O_2^{-}\bullet$ increases intracellular calcium and pH without altering viability of human amnion cells implying that it may serve an important biological function in these cells (Ikebuki *et al.*,1991).

Another important source of superoxide radical anions is from the metabolism of arachidonic acid. Indeed lipoxygenase enzymes have been shown to generate ROS (Halliwell and Gutteridge, 1990). This will be discussed further in chapter 8.
Nevertheless, it is generally agreed that the production of $O_2^{-}\bullet$ alone is insufficient to explain the cytotoxic and bactericidal activity of the respiratory burst (Das *et al.*, 1990). As already indicated, $O_2^{-}\bullet$ is the precursor of hydrogen peroxide, which is required in high concentrations before it can exert a bactericidal action. Thus $O_2^{-}\bullet$ must be a precursor of a more toxic species, the hydroxyl radical being the main candidate.

C. Hydroxyl radical (OH•).

The ability of this radical to react with numerous types of intracellular molecules makes it one of the most reactive chemical species known. It has a half-life of 9-10 seconds and has detrimental effects on most macromolecules (Sies *et al.*, 1992).
Radiolysis (X-rays, γ-rays) and photolysis of water are the major sources of the hydroxyl radical (OH•).

$2H_2O \rightarrow H_2O^{+} + e^{-} + H_2O^{*}$ (excited water molecule)

$H_2O^{*} \rightarrow H\bullet + OH\bullet$ (homolytic fission)

Other methods of OH• production require traces of catalytic transition metal ions, of which iron is the most important in vivo (Sutton and Winterbourn, 1989). This involves the univalent reduction of hydrogen peroxide by:-

(i) $O_2^{-}\bullet$, in the Haber-Weiss reaction

$$O_2^{-} + H_2O_2 \rightarrow O_2 + OH^{-} + OH\bullet$$

or

(ii) iron (or copper ions) in the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH\bullet$$

It is important to note that the exact chemistry occurring in vivo remains incompletely understood. Another means of producing the OH• is via a reaction with the nitric oxide radical (Roberfroid *et al.*, 1995).

$$H_2O_2 + NO\bullet \rightarrow HNO_2 + OH\bullet$$

Peroxynitrite, the other product, can oxidize SH-groups and propagate the formation of other noxious products, subsequently causing direct biological damage (Rubbo *et al.*, 1994).

The rate-constant for an uncatalysed Haber-Weiss reaction is very small but in the presence of oxidised metal ions OH• is rapidly generated. Consequently, it is vital to keep the concentration of free metal ions to a minimum. In physiological conditions, transferrin and ferritin proteins are involved in extracellular and intracellular storage of iron respectively. Ceruloplasmin, a specific copper-binding protein has an additional role in that it also prevents the initial formation of radicals by oxidising Fe^{2+} to Fe^{3+} (Halliwell, 1990). Considerable delerious effects can occur in certain pathologies when haemproteins are denatured, releasing free iron ions and generating OH•.

Many reactions involving OH• result in chain reactions, amplifying the initial effects of OH•. These involve either hydrogen atom abstraction or addition, thereby producing another radical species. The consequences of lipid peroxidation are devastating since cellular injury results from altered lipid fluidity and a loss of membrane integrity. The OH• radical abstracts a hydrogen atom from a methylene group of an unsaturated fatty acid, forming water and another radical. The lipid radical then takes up oxygen forming the peroxy radical and the propagation phase follows. Lower molecular weight hydroperoxides that are generated can diffuse through the membrane causing disturbances in other cellular functions away from the initial site of formation. Evidence supporting this has come from demonstrating a direct involvement of iron in lipid peroxidation (Borgi *et al.*, 1988). Lipid

peroxidation is recognised as an important factor in the progression of many chronic and degenerative diseases (Halliwell and Gutteridge, 1993).

Other major targets for OH• attack are the purine and pyrimidine bases of nucleic acids. Such reactions cause various modifications of the heterocylic bases via hydrogen addition reactions, leading to single and double strand breakages which can be mutagenic (Cochrane *et al.*, 1988).

1.3.3. Antioxidant mechanisms.

Halliwell and Gutteridge (1989) defined an antioxidant as any substance that when present at a low concentration, compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of that substance. Almost everything found in living cells, including proteins, lipids, carbohydrates and DNA, can be referred to as an "oxidizable substrate". From knowledge of the involvement of ROS in a number of pathologies (Winrow *et al.*, 1993), adequate mechanisms to protect cells from oxidative damage are essential.

In vivo an antioxidant can exert its action in a number of ways; by direct free-radical scavenging, by inhibiting ROS formation and/or by altering levels of endogenous antioxidant defences. Since many antioxidants have more than one mechanism of action, it is not surprising that the exact mode of action of these substances remains unclear.

The antioxidants have been divided into enzymatic and non-enzymatic, which will include both natural and synthetic types. Synthetic types will refer to biological antioxidants which have been chemically modified to generate synthetic compounds exhibiting novel properties. The list is extensive, but emphasis will be on a few examples and their principles of defence against ROS.

A. Natural and synthetic enzymatic antioxidants.

These are enzyme catalysts which remove ROS and prevent them from initiating a series of chain reactions. During the process they remain unconsumed. The three major antioxidant enzymes are the superoxide dismutases, catalase and glutathione peroxidases.

(i) Superoxide dismutases.

Natural superoxide dismutases are considered to be the first line of defence against $O_2^{-}\bullet$. Mammalian cells contain two types of SOD: the copper-zinc and the manganese enzyme (Beyer *et al.*,1988). These metalloproteins specifically catalyse the dismutation of superoxide radical anions to hydrogen peroxide and oxygen (Hassan *et al.*,1988).

$$O_2^{-}\bullet + O_2^{-}\bullet + 2H^{+} \rightarrow H_2O_2 + O_2$$

Due to the mounting evidence implicating $O_2^{-}\bullet$ in various pathologies, exogenously administered SOD have been tested as a form of therapy eg. in skin carcinogenesis. Synthetic SOD molecules are currently being engineered with higher catalytic rates than natural ones (Sies *et al.*,1991).

(ii) Catalase.

Catalase, a haem enzyme, is located mostly in intracellular peroxisomes. It protects the cell from hydrogen peroxide accumulation by converting it to water and oxygen (Bilinkski *et al.*,1988).

$$H_2O_2 \rightarrow 2H_2O + O_2$$

Many of the delerious effects of hydrogen peroxide are a consequence of its ability to diffuse across biological membranes readily. Synthetic catalase has been used to treat

conditions in which hydrogen peroxide has been implicated in detrimental effects, eg. inflammatory injuries.

(iii) Peroxidases.

Peroxidases catalyse the reaction below,

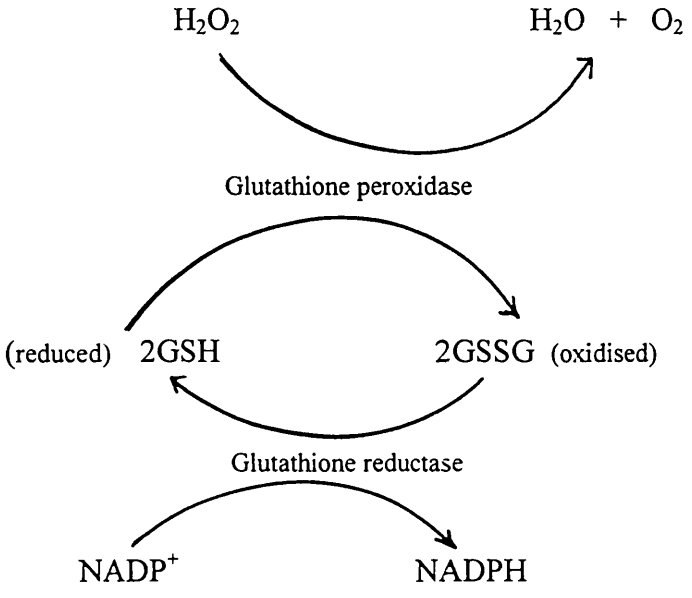
$$R\text{-}SH_2 \quad + \quad H_2O_2 \quad \rightarrow \quad R\text{-}S \quad + \quad 2H_2O$$

where $R\text{-}SH_2$ represents a reduced substrate.

Glutathione peroxidase is a selenium-dependent enzyme, present in high concentrations in the the cellular cytoplasm (Fantone *et al.*,1985). Its activity is dependent on the intracellular concentration of glutathione, glutathione reductase and NADPH (Chance *et al.*,1979). See Figure 1.13.

Figure 1.13

Pathways for the regulation of intracellular glutathione levels.



Glutathione peroxidase is effective at low hydrogen peroxide concentrations. Consequently, relative to catalase, glutathione peroxidase appears to play a more significant role in cellular protection against hydrogen peroxide-mediated damage. A synthetic equivalent to this enzyme, ebselen has been developed and is used to inhibit gastric and mucosal injury.

B. Natural and synthetic non-enzymatic antioxidants.

The strategies of defence employed by this group of antioxidants is usually determined by their ability to prevent lipid peroxidation or metal-catalysed radical reactions (Krinsky, 1992).

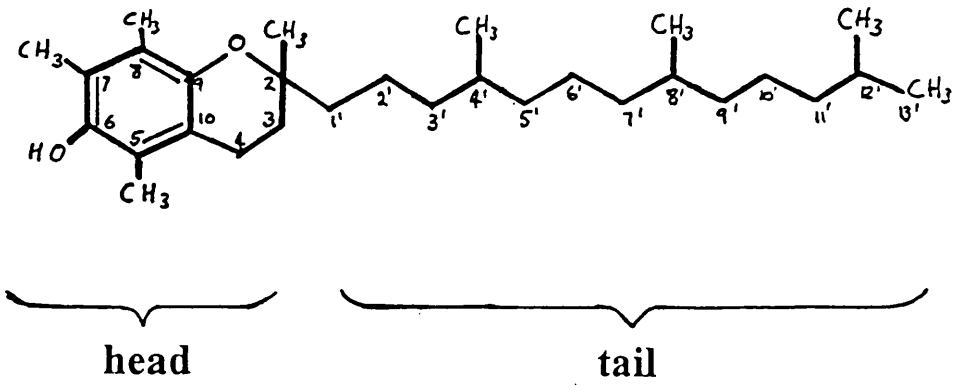
(i) Inhibition of lipid peroxidation.

(i) Tocopherols.

Vitamin E is the collective name for a group of naturally-occurring tocopherols found abundantly in plants, especially in plant oils (Packer, 1995). Tocopherols have a chromanol head group and a phytyl side chain ($C_{16}H_{33}$). Differing methyl substitutions around the aromatic ring of the head group determine the naming of the tocopherol, ie. α, β, γ or δ. See Figure 1.14.

Figure 1.14

Molecular structure of α-tocopherol.



The reactivity of α-tocopherol (3 methyl groups in positions 5,7 and 8), a major component of vitamin-E, with free radicals, is considered as its major biochemical function (Wefers and Sies, 1988). Vitamin E has been identified in relatively high concentrations in both cellular and mitochondrial membranes. The phytyl side chain favours its insertion into the lipid bilayer and the OH phenolic group is responsible for its antioxidant activity (Traber, 1995).

In vivo and in vitro studies with vitamin E have shown it to be a lipid soluble antioxidant, protecting the tissue lipids from free radical attack (Olson, 1992). It reacts with lipid peroxyl radicals forming vitamin E radicals. The unpaired electrons on the oxygen atom are delocalized into the aromatic ring structure making vitamin E radicals less reactive and more stable. Consequently, the radical chain reaction is terminated. Hence vitamin E is often referred to as a chain-breaking antioxidant. The stability of vitamin E radicals means they can be regenerated through a sequence of reactions involving ascorbic acid and $NADP^+$. Thus, the efficiency and lifetime of vitamin E as a biological antioxidant is determined by its rate of regeneration or recycling (Chan, 1992). See Figure 1.15.

Figure 1.15

Pathways for the oxidation and regeneration of vitamin E.

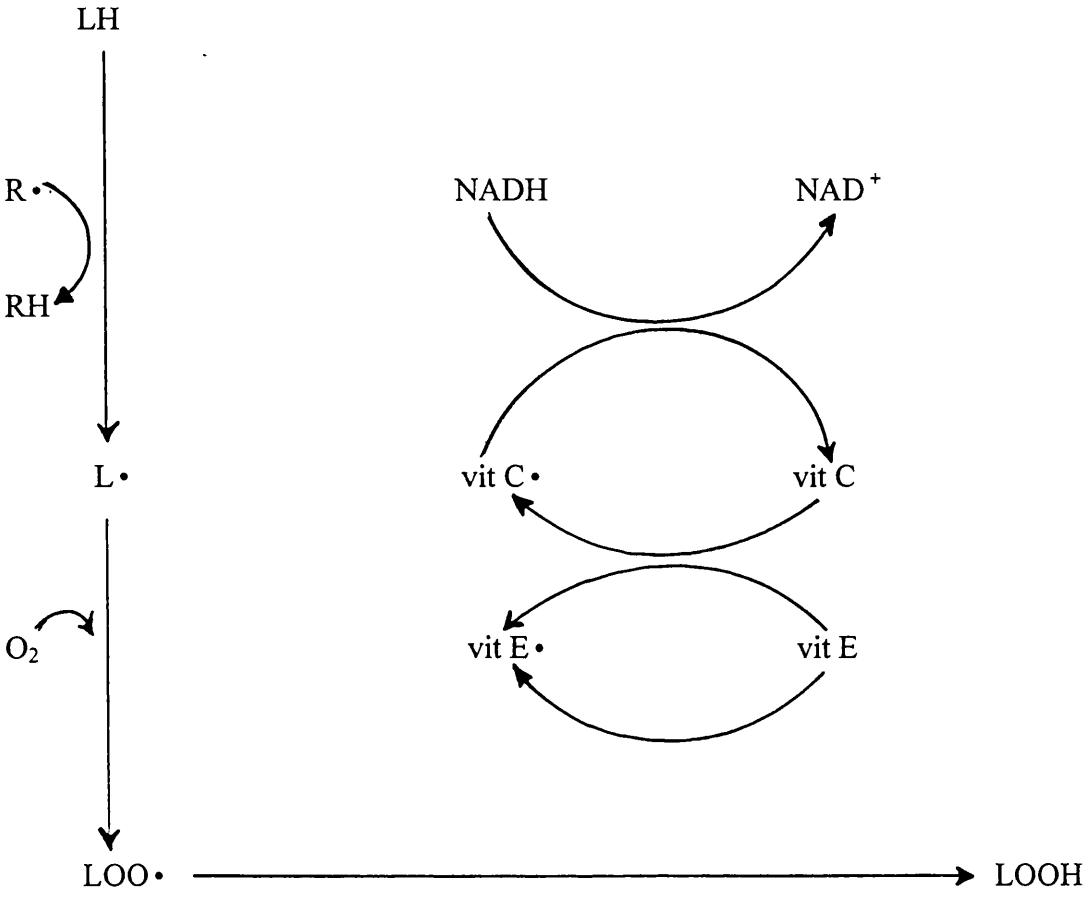


Figure 1.15 outlines the mechanism underlying lipid peroxidation and vitamin E recycling. R• indicates radical attack on a polyunsaturated fatty acid (LH). L• is a lipid radical, LOO• a lipid peroxyl radical and LOOH represents a lipid peroxide.

In addition to the antioxidant properties of vitamin E, it has been shown to have effects on cellular signalling and gene expression. Studies with vitamin E have demonstrated that not all its effects can be explained by its antioxidant properties. Work performed with vitamin E homologues have shown that α and β-tocopherol share radical chain breaking properties but have different actions on protein kinase C (Stauble *et al.*, 1994) and nuclear transcription factors such as AP-1 and NF-κB (Boscoboinik *et al.*, 1991). In both studies, the α form was more active than the β. The stage of the cell cycle seems to be important and its effects have been linked with the formation of an intermediate phosphorylatable protein where vitamin E acts as a ligand and protein modifier (Packer, 1995). In addition, it has been suggested that vitamin E exerts actions on the arachidonic acid pathway (Mahoney and Azzi, 1988). Much controversy surrounds the exact mode of action of vitamin E intracellularly, but is important to consider these points when examining the effects of vitamin E on cell proliferation.

Natural tocopherols have been modified to generate synthetic compounds exhibiting novel properties. Trolox is a water soluble derivative which is created by exchanging the phytyl side chain for a carboxylate group. However, to achieve greater similarity with in vivo models, vitamin E supplements are used. These contain eight stereoisomers, arising from the two possible orientations of each chiral centre. Only 12.5% of synthetic vitamin E is in the naturally occurring form (Traber, 1995). Differences in solubility of vitamin E in the lipid bilayer of membranes may be one explanation for the discrepancy seen between in vitro and in vivo results.

In a medical context, vitamin E supplementation is used in children with various clinical disorders, eg. cholestasis and α-betalipoproteinemia (Olson *et al.*, 1992) and is currently being examined in modulation of the immune function during AIDS (Wang *et al.*, 1995).

(ii) Ascorbic acid (vitamin C).

Ascorbic acid is another example of a radical chain-breaking antioxidant, yet unlike vitamin E it is water soluble. Humans are unable to synthesise ascorbic acid and require it from the diet. It has been located in cellular hydrophilic compartments (cytosol), plasma, synovial fluid and cerebrospinal fluid (Krinsky, 1992). In vivo and in vitro studies have shown it to function as a free radical scavenger and it is widely recognised to play a key role in protecting cells against oxidative damage. The interaction of ascorbic acid with $O_2^{-\bullet}$ has a faster rate constant than the vitamin E water soluble analogue, Trolox (Niki, 1991). In addition it readily reacts with aqueous peroxyl radicals responsible for attacking cellular membranes.

Mechanisms to regenerate ascorbic acid greatly enhance its antioxidant activity. Glutathione is vital in regenerating oxidised ascorbic acid back to ascorbic acid. Recent studies have indicated the existence of ascorbate transporters in neutrophils, which actively pump ascorbate against a concentration gradient from the plasma (micromolar range) into the cell (millimolar range). The exact relevance of this interesting mechanism remains to be established (Chan, 1992).

Additionally, ascorbic acid serves as a co-antioxidant via its interaction with vitamin E. Many studies have reported that it acts synergistically with vitamin E in preventing lipid peroxidation. It is proposed that due to ascorbic acid's water solubility it cannot suppress the oxidation of lipids resulting from the generation of radicals within the lipid bilayer itself. Vitamin E located in the lipid region performs this function, ultimately being consumed in the process. Ascorbic acid is thought to regenerate vitamin E and suppress oxidation for longer (refer back to Figure 1.15). Ascorbic acid has been shown to be more efficient than glutathione in its ability to reduce the vitamin E radical, via a redox transfer in the water-lipid interphase layer (Niki, 1991).

Paradoxically, under certain conditions ascorbic acid has been demonstrated to promote the generation of ROS (Stadtman, 1991). Ascorbic acid can reduce transition metals, Fe^{3+} or Cu^{2+}, allowing their participation in catalysed reactions producing $OH\bullet$ and $O_2^{-\bullet}$. However, extreme conditions in which iron is released from transferrin are needed before ascorbate-mediated damage can occur.

Natural ascorbic acid can be esterified with fatty acids such as palmitate to generate a more hydrophobic derivative whilst still retaining its functional antioxidant region. This change in solubility allows it to exert its action at different localisations in cells or fluids (Eriksson *et al.*,1994).

Much controversy exists over claims that vitamin C can prevent or cure the common cold . Johnson, C. J (1992) suggested that this action of vitamin C could be related to a number of its properties eg. providing protection against the $O_2^{-\bullet}$ produced by neutrophils, regeneration of vitamin E and/or its action as an antihistamine.

Recently ascorbic acid has been tested in numerous studies for its action on the growth of the acquired immune deficiency syndrome (AIDS) virus (Harakeh *et al.*,1994; 1991).

(iii) Glutathione.

Glutathione (GSH) is a cysteine containing tripeptide (γ-glutamyl-cysteinyl-glycine) that is found in millimolar concentrations in eukaryotic cells (Staal *et al.*,1992). One of its many functions includes cellular defence against intracellular reactive intermediates (Orrenius *et al.*,1988). It participates directly in the destruction of ROS via a one electron oxidation reaction, forming thiyl radicals, and also acts as a cofactor of several protective cytosolic and membrane bound enzymes eg, peroxidases (Meister, 1983) (refer back to Figure 1.13). This dual role is owed to the thiol function.

GSH is a cofactor for the reductase which recycles dehydroascorbate back to ascorbic acid (Meister *et al.*,1983). A number of studies have also suggested that GSH is vital in the maintenance of α-tocopherol in the reduced state (Wefers and Sies, 1988).

GSH detoxifies hydrogen peroxide very effectively in the glutathione peroxidase catalysed reaction:

$$H_2O_2 + \underset{\text{reduced}}{GSH} \rightarrow H_2O + \underset{\text{oxidised}}{GSSG}$$

However, its efficacy depends on the availability of intracellular GSH and the cell's ability to reduce glutathione disulphide (GSSG). This reaction is catalysed by glutathione reductase, and NADPH levels are maintained by a pentose phosphate shunt (Forman, 1990).

$$NADPH + GSSG + H^+ \rightarrow 2GSH + NADP^+$$

GSSG itself is a relatively strong oxidant that reacts readily with protein thiols, forming mixed disulphide proteins (proteinS-SG). This may modify protein conformation and consequently protein activity. Thus, these potentially damaging effects of thiyl radicals and their derivatives cannot be ignored (Halliwell, 1990).

Another important feature of GSH is its low molecular weight allowing it to traverse gap junctions. This may explain why GSH-deficient cells can be protected from irradiation when in contact with normal cells (Kavanagh *et al.*,1988).

However, when GSH is added extracellulary it does not penetrate cells well. Glutathione ethylester has been synthesised as a precursor which penetrates into cells and is then hydrolysed to GSH (Meister and Anderson, 1983). Another method of increasing intracellular GSH levels is by supplying substrate, thus supporting GSH biosynthesis. N-acetylcysteine (NAC) is a cysteine-containing precursor. NAC has a dual antioxidant action; it can restore intracellular GSH and also scavenge oxidants directly. Recently, studies with NAC have demonstrated that it can enhance T cell mitogenesis, IL-2 production and T cell growth in culture (Eylar *et al.*,1993). The exact mechanism by which low NAC concentrations enhance IL-2 release in T cells remains unclear. There are numerous transcription factors which regulate the IL-2 gene and activation is thought to require co-ordination of all of them. It is proposed that some factors could be sensitive to GSH levels whilst others are sensitive to ROS. The exact relationship between GSH and ROS (and NAC indirectly) is a complex but important consideration when interpreting in vitro results with NAC.

Lymphocytes from AIDS patients are thought to be deficient in GSH, thus making them more susceptible to damage from oxidative stress: NAC has been proposed as a potential form of therapy (Simon *et al.*,1994; Staal *et al.*,1990; Roederer *et al.*,1990).

(ii) Removal of catalytic metal ions.

Metal chelators function as antioxidants by decreasing the concentration of free metal ions that catalyse reactions generating ROS. The removal of iron and copper ions is particularly important. As free ions, these metals can catalyse the Haber-Weiss reaction and participate in the Fenton reaction, generating the powerful OH•, or reducing organic hydroperoxides (LOOH) (Krinsky, 1992).

$$LOOH + Fe^{2+} \rightarrow LO + OH^{-} + Fe^{3+}$$

It is vital to regulate the concentration of "free " iron, since extracellular body fluids contain little or no catalase activity and extremely low levels of SOD (Halliwell and Gutteridge, 1990). Transferrin, an iron-binding protein prevents ferrous (Fe^{2+}) iron from participating in radical reactions (Gutteridge *et al.*,1981). Other examples of metal binding proteins include lactoferrin made by neutrophils (Gutteridge, 1981) and the copper binding protein ceruloplasmin (Halliwell, 1994). It is speculated that GSH may also be able to complex copper ions (Roberfroid, 1995).

Iron has been implicated in the pathology of β-thalassaemia in which the availability of free iron is increased (Santos *et al.*,1994). Hence, iron chelators, desferrioxamine (Desferal) can be therapeutically used to complex iron.

Desferrioxamine is also widely used as an iron chelator in in vitro studies. However, its exact mechanism of action is debatable (Morel *et al.*,1992). Although it is well documented that desferrioxamine has a high stability constant with ferric iron, its action in vitro may be exerted via different mechanisms. Possible explanations are that it may have an inhibitory action on enzymes which use iron as a co-enzyme (eg. ribonucleotide reductase, thus inhibiting DNA synthesis) or inhibit free radical production directly (Carotenuto *et al* ,1986).

In addition, iron is also important as a signalling molecule (Gutteridge, 1994). Iron is delivered to the cell via the transferrin receptor, whose synthesis rate is regulated at the post-translational level. Iron acts as a signal to regulate the synthesis of the transferrin receptor. Another study has shown that iron may influence the expression of T lymphocyte surface markers (CD4 and CD2 down-regulation) which would ultimately affect T cell recognition, positioning and activation (Santos *et al.*,1993). Thus, desferrioxamine may influence T cell signalling indirectly.

As described above, there are a range of likely mechanisms whereby ROS, if present to excess, could influence cellular function. In an attempt to protect the cell against oxidative damage, cells utilise a series of antioxidant compounds as discussed. Whereas the deleterious effects of ROS are well-characterised, there is now a growing body of evidence to suggest that ROS could play a crucial role in T cell proliferation and possibly function as "cellular messengers". This will be examined in the next section.

1.4 Redox regulation.

Earlier, the potential origins of ROS and the damaging consequences of excess ROS production were outlined. The redox state of a cell is a balance between the reductive species, such as glutathione, and the oxidative species, such as ROS. Ultimately the concentration of ROS determines the cells proliferating capacity. This section will cover the action of small quantities of ROS, and their influence on signalling functions cited in section 1.2.

1.4.1 Cellular redox status.

Current evidence suggests that most biochemical events critical for cell proliferation, occur in the period between mitotic division and the time during which the DNA is replicated (S phase) (Pardee, 1989).

Recently it has been suggested that small, nontoxic amounts of ROS may play a crucial role in the mechanisms underlying proliferative responses. A great variety of agents have been shown to generate ROS. These include phorbol esters, which can activate NADPH oxidase and ultimately the respiratory burst (Rabesandratana *et al.*, 1991; Whitacre *et al.*, 1991), TNF, IL-1 (Feng *et al.*, 1995) and CD28 (Los *et al.*, 1995a). Furthermore, ROS are small, diffusible molecules which can be rapidly synthesised and destroyed, thereby fulfilling an important criterion as an intracellular messenger (Schreck and Baeuerle, 1991). In addition, low concentrations of hydrogen peroxide are potent activators of T lymphocyte functions, early expression of IL-2 and IL-2 R α chain genes (Los *et al.*, 1995; Sekkat *et al.*, 1988). Numerous studies have shown that antioxidant compounds can inhibit T cell proliferation (Chaudri *et al.*, 1986; 1988; Novogrodsky *et al.*, 1982; Dornand and Gerber. 1989; Fedyk *et al.*, 1994; Hunt *et al.*, 1991).

In contrast to bacteria, mammalian cells do not have specific sets of genes which are activated in response to ROS (Demple *et al.*, 1991). Thus, the mechanisms by which ROS are sensed are less well understood. It could be that ROS modulate the efficiency of the overall process of signal transduction at many sites. The cell's redox

status is mainly buffered by GSH, coupled to the oxidation state of cysteine residues by a complex mechanism involving thiol/disulphide exchange (Staal *et al.,* 1994).

T lymphocytes have a very low transport activity of L-cysteine (precursor of GSH), thus they are highly susceptible to changes in the intracellular redox state. Decreases in the intracellular GSH level can lead to alterations in the activity of redox-sensitive enzymes. ROS can influence GSH levels through the activity of glutathione peroxidase, generating GSSG and ultimately altering the GSH:GSSG equilibrium. Hence, it is possible that ROS-induced alterations in tyrosine phosphorylation (Staal *et al.,* 1993), protein kinase activation (Schrieven *et al.,* 1993), in particular PKC, (Staal *et al.,* 1990) and *ras* activation (Lander *et al.,* 1995) are indirectly related to its influence on GSH levels.

Although protein kinases, phosphatases and G proteins play a pivotal role in intracellular signal transduction, another group of molecules central to the regulation of signal induced gene responses are the nuclear transcription factors.

1.4.2. Redox-sensitive proteins.

A. NF-κB.

NF-κB was the first eukaryotic factor shown to respond directly to ROS (Schreck *et al.,* 1991).

NF-κB is present in the cytoplasm as an inactive complex bound to an inhibitory subunit, IκB. The active form of NF-κB are dimers composed of two DNA binding subunits, 50kDa and 65kDa (RelA). T cell activation results in the rapid release of IκB, allowing NF-κB to translocate to the nucleus and bind to its DNA sequences (Baeuerle and Henkel, 1994).

NF-κB plays a key role in the regulation of numerous genes involved in pathogen responses and cellular defence mechanisms. These include the genes for cytokines (IL-2), immune receptors and acute phase proteins (Molitor *et al.,* 1990). Consequently, it is thought that NF-κB is specialised as a signal transducer and mediator of the immediate early phases of the immune response.

An intriguing feature of NF-κB is that it can be activated by a large variety of unrelated stimuli. These include PMA, TNF, IL-1 and CD28 (Bomsztyk *et al.*, 1991). One common feature of all these inducers is their ability to induce ROS formation. In support of a ROS-mediated activation of NF-κB, hydrogen peroxide can also cause NF-κB activation (Schreck *et al.*, 1991). Furthermore, activation of NF-κB by all stimuli known to date is blocked by antioxidant compounds (Schreck *et al.*, 1992; Toledano *et al.*, 1991). The precise mechanism by which ROS activate NF-κB is unclear. A recent study has shown that the phosphorylation of IκB can be blocked by antioxidant compounds. Consequently, an IκB kinase could be activated or, an IκB phosphatase inactivated (Schulze-Osthoff *et al.*, 1995). Since hydrogen peroxide can cause the tyrosine phosphorylation of multiple proteins, it is speculated that the intracellular redox status controls NF-κB activation by indirectly regulating tyrosine phosphorylation (Goldstone *et al.*, 1995; Anderson *et al.*, 1994).

B. AP-1.

Redox regulation of DNA binding, via a cysteine residue, has been implicated for the transcription factor AP-1 (Xanthoudakis *et al.*, 1992).

AP-1 can exist in two forms; either as a heterodimer of c-jun and c-fos proteins or, a c-jun homodimer. Both forms bind the so called PMA-responsive element (section 1.2.5.C) and are responsible for the transcriptional induction of a number of genes in response to PKC activation (Karin and Smeal, 1992). Like NF-κB, PMA induces AP-1 activation. PMA-mediated activation relies on a nuclear phosphatase which activates pre-existing c-jun homodimers (Meyer *et al.*, 1994).

The actions of hydrogen peroxide on AP-1 are contrasting to those of NF-κB. Hydrogen peroxide induces only a weak stimulation of AP-1-dependent gene expression. Furthermore, high levels of AP-1 activity following PMA stimulation are significantly decreased after hydrogen peroxide addition, suggesting that PMA activation of AP-1 uses a different mechanism of activation to that of NF-κB. Antioxidants potentiate PMA-mediated AP-1 activation through a synergistic

mechanism (Meyer *et al.*, 1993; Schenk., 1994). Thus, NF-κB is proposed to be an oxidative stress-responsive factor and AP-1 an antioxidant-responsive factor.

AP-1 requires new protein synthesis for activation, which is stimulated under both oxidant and antioxidant conditions. However, although the transcription of c-fos and c-jun genes is redox regulated (Abate *et al.*, 1990), increases in these genes do not correspond to the induction of AP-1-DNA binding. Hence, it is speculated that a highly active form of AP-1 protein is only produced under antioxidant conditions. The exact implications of this hypothesis are still unclear, but they do demonstrate the existence of redox-regulated transcription factors. It could be that early T cell responses are favoured in an oxidant environment, but that later responses require the cellular conditions to return to normal and even antioxidant, favouring optimal AP-1 activation (Schulz-Osthoff *et al.*, 1995).

C. *Ras*

Recently, *ras* has been identified as a central target by which the nitric oxide radical transmits signals (Lander *et al.*, 1996). Hence, it is possible that this low molecular weight G-protein maybe a more general target for ROS and senses the cellular redox status. Indeed another study by Lander *et al* (1995) has shown that MAP kinases immunoprecipitated from cells treated with hydrogen peroxide had an enhanced ability to phosphorylate, compared to cells untreated with hydrogen peroxide.

Clearly, interest in *ras* as a "target" for oxidative signalling ultimately generating a nuclear signal, is a field of great interest.

In conclusion, ROS may interact directly with protein kinases or transcription factors/inhibitors through oxidative mechanisms. In addition, it is also possible that ROS could indirectly modulate the redox state and activity of these signalling proteins through changes in the cellular levels of GSH and GSSG, and ultimately redox-sensitive enzymes. Whether ROS act as intracellular signalling molecules or regulate cell cycle entry by indirect means remains to be established.

1.5 Aims of the project.

The initial aim of the project has been to determine whether T cells are able to generate a ROS signal. To accomplish this, lymphocytes in the presence and absence of accessory cells were studied. ROS generation was measured using a free radical sensitive dye, 2'7'-dichlorofluorescein, by flow cytometry.

Previous studies with lymphocytes have mainly focused on examining free radical generation stimulated by the phorbol ester, PMA, by lymphocytes in a mixed cell population. Less, however, is known about the activation events induced by a combination of stimuli acting in synergy for proliferation and IL-2 release. A number of compounds known to act in synergy for proliferation and IL-2 release were examined for ROS production.

In order to test the theory that oxidants directly activate redox-sensitive "receptors", a range of ROS scavengers, with differing modes of action, were used. In order to elucidate the underlying relationship between ROS and subsequent T cell activation, the sensitivity of ROS, IL-2 release and proliferation to these antioxidant compounds was compared.

As discussed in section 1.6.2, the CD2 $T11_1$ epitope has been suggested to mediate a negative signalling pathway for T cell activation. To investigate the role of ROS in this pathway, the latter part of the project studies the influence of anti-CD2 on two main lymphocyte functions: ROS production and proliferation.

CHAPTER 2

METHODS AND MATERIALS

2.1 Materials.

A list of all the chemicals/reagents used in this project is provided in Table 1.1. Antibodies received as gifts are acknowledged with the name and address of the supplier.

All reagents were of the highest grade commercially available. Where work involved cell lines or human blood cells, the equipment used was pre-irradiated and of a disposable nature (eg. flasks and pipettes); otherwise it was autoclaved and sterility validated with a Browns tube. Sterile cell culture flasks and 96 well round and flat-bottomed plates were Falcon products purchased from Marathon Laboratories. The 12ml centrifuge tubes were bought from Alpha Laboratories and the 50ml centrifuge tubes were made by Nunc (Gibco Life Science Technologies). Disposable pipettes were from Philip Harris.

2.2 Safety and sterility procedures.

The Ethics Committee of UCL gave approval for this work.

All manipulations involving the use of cell lines or human cells were performed in an Astecair BHA 48 Class 2 safety cabinet, using aseptic techniques.

Following preparation of cultures under sterile conditions, all short and long term incubation periods were carried out at a temperature of 37°C in an atmosphere containing 95% air and 5% carbon dioxide.

Table 2.1 **List of chemicals/reagents**

CHEMICAL/DRUG	NAME OF SUPPLIER/ADDRESS
A23187	Sigma, Poole, GB.
anti-CD2 mAb OKT11	Dr. P. Beverly, ICRF, UCL.
anti-CD3 mAb $UCHT_1$	Sigma Immunochemicals, Poole, GB.
anti-CD14 mAb UCHM1	Dr. P. Beverly, ICRF, UCL.
anti-CD19 mAb BU12	Dr. P. Beverly, ICRF, UCL.
anti-CD28 mAb 913.12	Serotec, Oxford, G.B.
anti-CD28 mAb 15E9	Dr. P. Beverly, ICRF, UCL.
anti-MHC Class II (L243)	Dr. M. Binks, Dept Immunology, UCL.
Ascorbic acid	Sigma, Poole, G.B.
Catalase	Sigma, Poole, G.B.
Desferrioxamine	Ciba-Geigy, Horsham, G.B.
2'7'-dichlorofluoroscein (DCFH-DA)	Molecular Probes, Cambridge, G.B.
Dimethyl sulfoxide (DMSO)	Sigma, Poole, G.B.
Dynabeads M-450 Sheep anti-Mouse IgG	Dynal Ltd, Meryside, G.B.
Ethlenediaminetetraacetate (EDTA)	Sigma, Poole, G.B.
Foetal calf serum (FCS)	Gibco Life Science Technology, G.B.
FITC-labeled rabbit (anti-mouse) IgG	Dako, High Wycombe, G.B.
Hank's balanced salt solution (HBBS)	Gibco Life Science Technology, G.B.
Histopaque	Sigma, Poole, G.B.
Human recombinant IL-2	Sigma Immunochemicals, Poole, G.B.
Mimimum Essential Medium (MEM)	Gibco Life Science Technology, G.B.
N-acetyl cysteine	Sigma, Poole, G.B.
Phorbol myristate acetate (PMA)	Sigma, Poole, G.B.
Roswell Park Memorial Institute Medium	Gibco Life Science Technology, G.B.
[^{3}H]-thymidine	Amersham International plc, G.B.
Vitamin E (α-tocopherol)	Sigma, Poole, G.B.

2.3 Cell culture media.

Cells were grown in Roswell Park Memorial Institute medium-1640 (RPMI-1640), which was supplemented with 2 x 10^{-3} M glutamine, 10% v/v heat inactivated foetal calf serum (FCS) and antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml fungizone).

RPMI was also used to dilute any drugs which were to be added to cell cultures.

Hank's Balanced salt solution (HBBS) supplemented with 10% v/v FCS and phosphate buffered saline (PBS) were used during the preparation of purified T cells and in flow cytometric assays.

2.4 Cell counting.

Samples were counted using an improved Neubauer haemocytometer. 50μl of the cell suspension was mixed thoroughly with an equal volume of trypan blue dye. A 20μl sample was then used to count the cell density. Trypan blue is excluded by live cells but not by dead or dying cells, therefore this staining also gives an assessment of cell viability. Samples were only accepted for use if 90% or more cells were viable.

2.5 Isolation of human peripheral blood mononuclear cells.

Typical yields obtainable by this method were 1-1.5 x 10^8 peripheral blood mononuclear cells (PBMC) from 120ml of whole blood.

120ml of venous blood was obtained from healthy volunteers and mixed immediately with 108ml sterile distilled water, 12ml PBS (x10 concentration) and 4ml 0.1M ethlenediaminetetracetate (EDTA) to prevent coagulation. 18ml of the diluted blood was carefully layered onto 12ml ficoll-hypaque (histopaque) with a specific gravity 1.077g/ml. 12-16 50ml centifuge tubes were prepared in this manner and spun at 400g for 35 minutes at room temperature (25°C).

The PBMC layer (visible as a "buffy" layer between the plasma and histopaque) was carefully aspirated. The cells were washed twice with 10ml Minimum Essential Medium (MEM) containing 25mM HEPES (4(-2-hydroxyethyl)-piperazine ethanesulphonic acid). Subsequent "washing" of cells will refer to the suspension of

cells in 10ml of media which was centrifuged at 400g for 6 minutes, unless otherwise stated. Supernatants were discarded and the cells resuspended in a known volume of medium for cell counting.

Cell density was adjusted so that a final cell density of 2 x 10^6 cells/ml was present in each sample after the addition of all drugs.

2.6 Proliferation and IL-2 assays: addition of drugs.

Proliferation and IL-2 assays were performed in 96 well microtitre plates, which held a final volume of 200µl/well. Each experimental point was set up in triplicate and each protocol was repeated at least three times. Controls which did not contain a particular compound were supplemented with an equivalent volume of RPMI-1640.

Phorbol myristate acetate (PMA), calcium ionophore A23187, vitamin E and 2'7-dichlorofluoroscein (DCFH-DA) are lipophilic and were dissolved in ethanol or dimethylsulfoxide (DMSO) before being diluted with RPMI or PBS. The concentration of DMSO applied to cells did not exceed 0.025%. The DMSO at this concentration was without effect on either PBMC or the CTLL cells, assessed by the trypan blue exclusion method. All drugs and solutions were prepared fresh on the day of the experiment. Other compounds used were water soluble and could be directly dissolved in RPMI or PBS.

Drug concentrations were adjusted to allow for dilution with other compounds which were also being added to the sample wells. Each plate contained the following:

-50µl cell suspension. PBMC were used at 8 x 10^6 cells/ml and purified T lymphocytes at 4 x 10^6 cells/ml (final concentration in each well was 2 x 10^6 cells/ml and 1 x 10^6 cells/ml respectively).

-50µl medium or antioxidant (4 times the final concentration required).

-100µl medium or stimulants. Where a combination of two stimulants was used each was added at 4 times the final concentration required.

-Antioxidants (desferrioxamine, ascorbic acid, DMSO, N-acetyl cysteine and vitamin E) were present in the cultures throughout the experiment (48 hours). The

absence of toxicity at the concentrations used was verified by the trypan blue exclusion method.

Since the time course of the formation of reactive oxygen species (ROS) is very rapid a different experimental protocol was followed which will be discussed later.

2.7 Assessment of the G_1 phase of the cell cycle:

Assay of IL-2.

The IL-2-dependent murine cytotoxic T lymphocyte line (CTLL) was used as indicator cells. CTLL proliferate in the presence of IL-2 but accumulate in early G_1 and undergo apoptosis without the lymphokine. CTLL are unusual in that they cannot enter Go or a quiescent state. Thus, from this knowledge, and providing that the number of cells in any one test remains constant, the number of viable cells after a given period of time will be proportional to the IL-2 concentration in the sample medium.

2.7.1 CTLL cell line maintenance.

CTLL were grown in supplemented RPMI, and were subcultured to ensure that the cell density did not exceed 8×10^5 cells/ml. On Mondays and Wednesdays CTLL cells were adjusted to a density of 2×10^4 cells/ml in fresh RPMI containing 15-20 U/ml of human recombinant IL-2 (r-IL-2). On Fridays cells were subcultured into fresh RPMI but at a lower density for 3 days (1×10^4 cells/ml). CTLL were frequently monitored in between subculture days to check that the cells were not overgrowing or dying.

The timing and cell density protocols for optimal CTLL growth and maintenance was that found by Dr. B. M. Chain, University College London.

2.7.2 Harvesting of PBMC supernatants.

Following completion of the 48 hour culture period, microtitre plates containing PBMC were centrifuged at 300g for 5 minutes to pellet the cells. 100µl of supernatant were removed from each well using a multichannel dispenser into a copy plate. The

supernatants in the copy plate were frozen overnight and kept at -20°C until CTLL had reached optimal density. Freezing the plates also destroys any live PBMC which may have been carried over.

2.7.3 Preparation of CTLL for the IL-2 assay.

CTLL had to be washed twice in MEM to remove any IL-2 from the culture medium. After the second wash they were resuspended in fresh RPMI and incubated for a minimum of 1 hour at 37°C to remove any IL-2 bound to the cell surface receptor. This was an important step to perform in order to minimise background interference of the assay. After the one hour incubation, cells were washed twice more in MEM before being resuspended in RPMI at a density of 1×10^5 cells/ml.

2.7.4 Bioassay for IL-2.

Dilutions of the IL-2 containing supernatants were made to achieve a 1 in 8 dilution after the addition of CTLL. Each well contained 12.5µl of the supernatant, 37.5µl of RPMI and 50µl of the CTLL suspension (5000 cells per sample well). Plates were stored at 37°C for 25 hours. The cell viability was dependent on the amount of "growth promoting" IL-2 present in each sample well. 10µl of a solution of [^{3}H]-thymidine (100µCi/ml) was added to each well. Cells were harvested after a further 24 hours and the radioactivity of each sample determined by liquid scintillation counting.

2.7.5 Interpretation of the CTLL assay.

Background counts refer to the [^{3}H]-thymidine incorporated by CTLL in the absence of IL-2, ie. grown in RPMI medium only. This value was subtracted from the counts per minute (cpm) of all other wells. The samples are expressed as a % of their control values (normally stimulated cells in the absence of antioxidant):

$$\frac{\text{stimulated cells with drug - background counts}}{\text{stimulated cells without drug - background counts}} \times 100$$

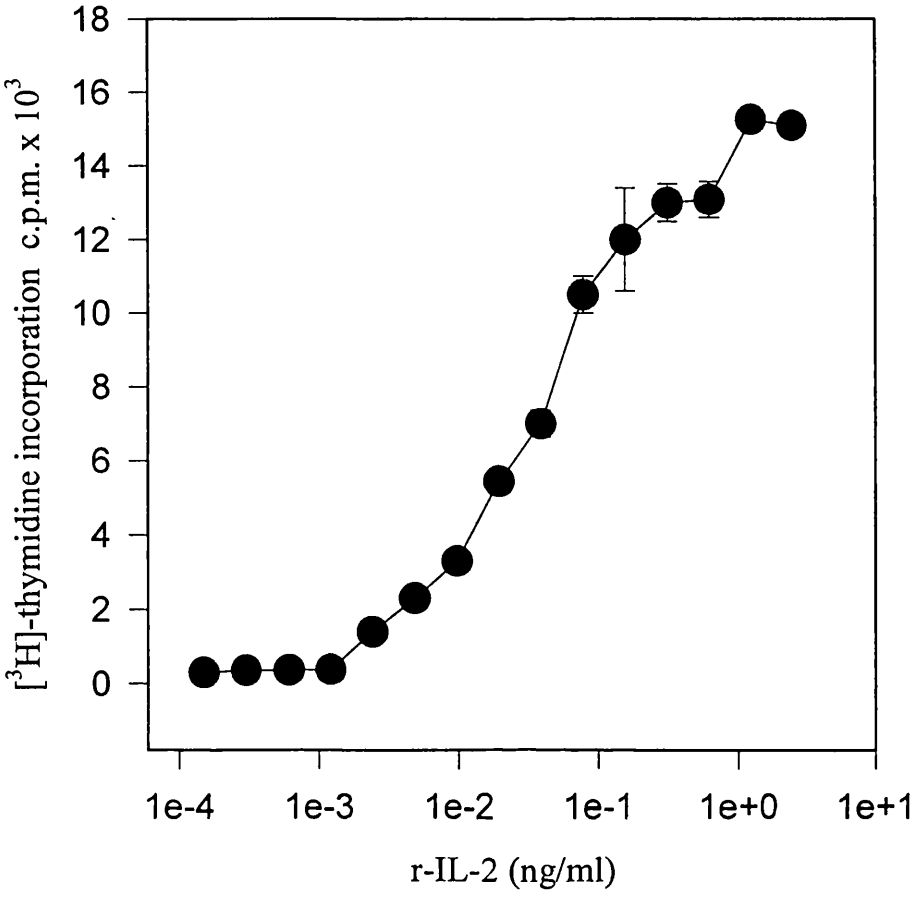


Figure 2.1
A typical standard curve displaying the effect of IL-2 on the viability of CTLL cells.
CTLL were incubated for 25 hours with r-IL-2 at the concentrations shown. [^{3}H]-thymidine was then added and the cells incubated for a further 24 hours. Cell viability was determined by β liquid scintillation spectroscopy. The symbols represent the mean and vertical bars of the s.e.m. from one experiment performed in triplicate. Where error bars are not visible they are contained within the limits of the symbol.

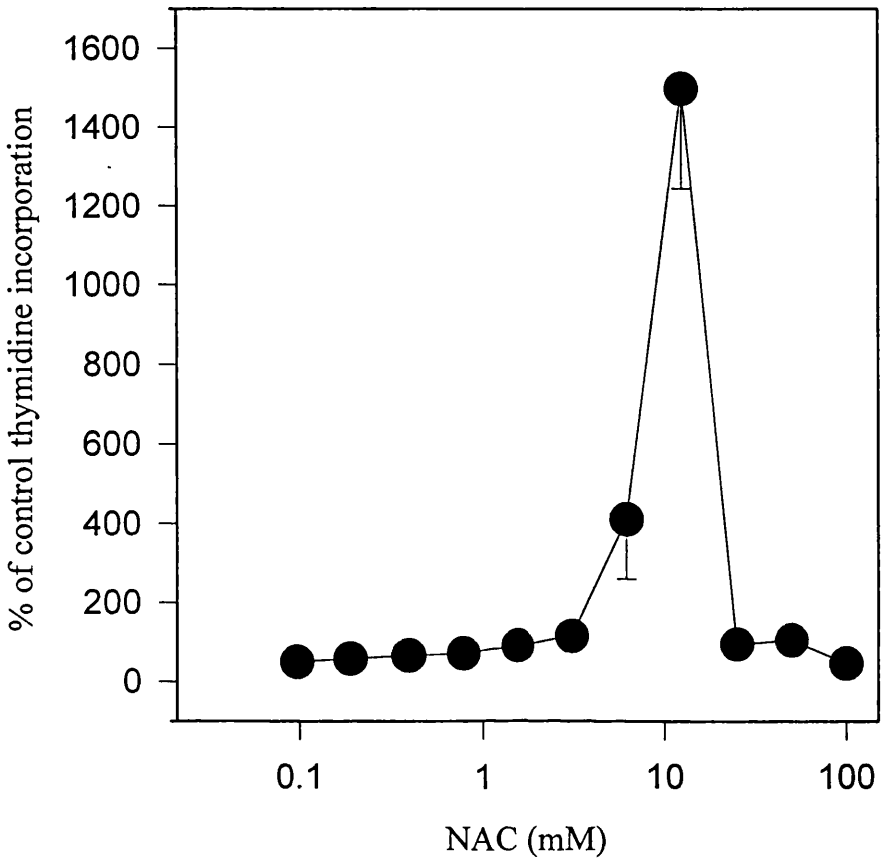


Figure 2.2
The effect of NAC on CTLL growth.
The concentration-response relationship for the effect of NAC on CTLL viability in the presence of a submaximal concentration of human r-IL-2, 0.156ng/ml. CTLL were treated with NAC at the concentrations shown; r-IL-2 was added simultaneously. After 25 hours of incubation a pulse of [^{3}H]-thymidine was added and the cells incubated for a further 24 hours before harvesting. Results are expressed as the % of the thymidine incorporated by CTLL in the control sample containing no NAC, which was 6500 ± 200 cpm. The symbols represent the mean of triplicate samples in one experiment and the vertical bars are s.e.m. Where bars are not visible they are contained within the limits of the symbol.

2.7.6 Controls.

It was essential to ensure that diluted antioxidants had no effect on the CTLL cells, which could be attributed incorrectly to changes in IL-2 concentrations. Hence the direct action of antioxidants on CTLL growth was examined in the presence of a known concentration of r-IL-2. Samples of r-IL-2 for IL-2 standard curves and for use in antioxidant control experiments were gifts from Dr. B.M. Chain, Department of Immunology, UCL. Standard curves were performed to determine whether the CTLL were responding to IL-2 concentrations, ie. dying at low concentrations and proliferating at optimal levels of IL-2. A typical standard curve is shown in Figure 2.1. All drugs and antioxidants used had no significant effect on CTLL growth. An exception however was NAC (Figure 2.2). NAC alone had a biphasic action on CTLL growth in the presence of 0.156ng/ml r-IL-2. Hence, CTLL could not be used to determine IL-2 release from stimulated PBMC treated with NAC.

All IL-2 assays were performed in round bottomed 96 well microtitre plates.

2.8 Assessment of S phase : Proliferation assay.

This assay uses thymidine labelled with the hydrogen isotope tritium, which when added to cultures becomes incorporated into newly synthesized DNA. The thymidine is labeled on the 5-methyl group and the level of radioactivity is proportional to the amount of new DNA synthesis and therefore cellular proliferation.

Cells were pulsed with 10µl of a solution of [^{3}H]-thymidine (100µCi/ml) for the last 16 hours of a 48 hour culture. Microtitre plates were centrifuged at 300g for 5 minutes. Supernatants were removed (used for IL-2 assay) using a multichannel dispenser. The remaining 100µl of supernatant, containing the cell pellet, was transferred onto glass fibre paper using a Skatron AS cell harvester. The filters were placed into pico pro scintillation vials and 2ml of Instagel scintillation fluid added.

Each sample was counted for 3 minutes on a Packard Tri-carb 4660 scintillation counter. Results were given as counts per minute (cpm).

Previous work has often proposed that it is possible to freeze plates once the culture period is complete and harvest at a later stage. However, this is not good practice

unless all experiments are performed in the same way. Freezing plates generates lower counts which could be misinterpreted when analysing the data. Furthermore, freezing plates introduces an unnecessary variable into the whole experimental protocol (Sabbe *et al.*, 1983).

2.9 Preparation of purified T lymphocytes.

This technique relies on the matrix design of Dynabeads M-450 Sheep anti-Mouse IgG, which makes them suitable for immunomagnetic separation. Dynabeads M-450 Sheep anti-Mouse IgG will be referred to as simply Dynabeads.

Dynabeads are of optimal size (diameter: 4.5μM) for cell isolation and have high affinity for purified sheep anti-mouse IgG covalently bound to the surface. The targeted rosetted cells can be easily isolated by applying a magnet on the wall of the test tube. In this purification procedure, the cells targeted and rosetted for removal expressed major histocompatibility (MHC) class II molecules on their surface, functionally defined as antigen-presenting cells. They include dendritic cells, macrophages, monocytes and B cells.

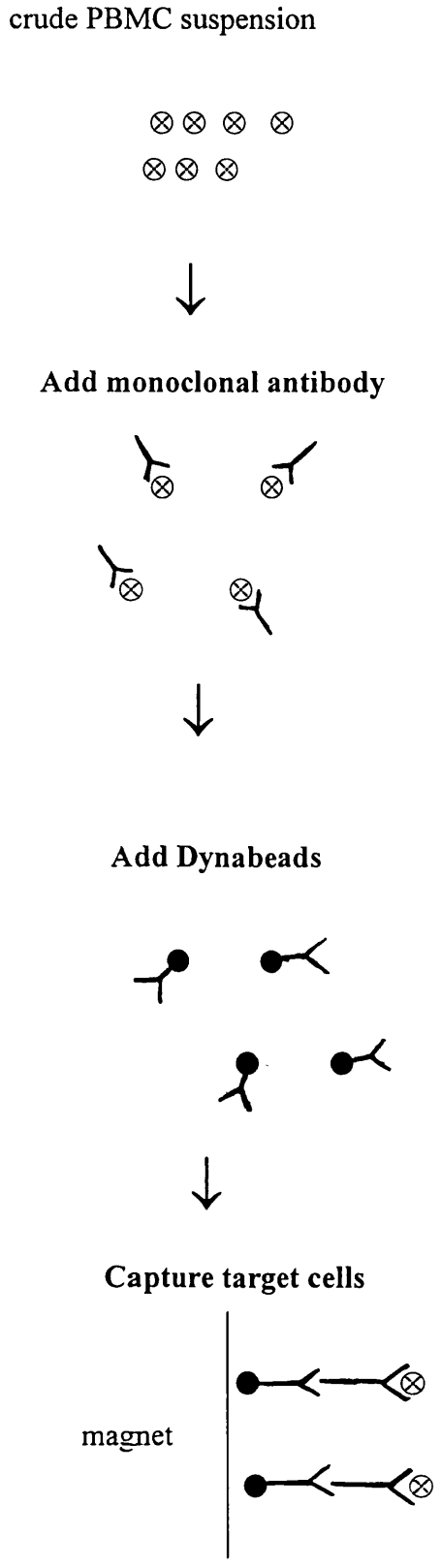
Optimization of cell yield is dependent on two main variables; the number of beads used and the incubation time. Dynal Laboratories recommend the use of 1-2 x 10^7 beads/ml per sample (concentration of vial purchased: 4 x 10^8 beads/ml), 4 beads per target cell to be removed, and an incubation time of 10-30 minutes.

An indirect technique was used for the preparation of purified human T lymphocytes from the "buffy layer" of PBMC, isolated from venous blood as previously described.

2.9.1 Indirect technique strategy.

In the indirect technique the cells are first incubated with a specific primary antibody against CD14, CD19 and MHC class II molecules, and subsequently rosetted with the Dynabeads. See Figure 2.3.

Figure 2.3 Dynabead indirect technique strategy



2.9.2 Cell preparation.

PBMC were plated onto plastic petri dishes and incubated for 2 hours at 37°C to remove monocytes. The cells were carefully removed using a sterile pipette and resuspended at 1 x 10^7 cells/ml in Hank's Balanced Salt solution (HBBS) supplemented with 5% FCS. Monoclonal antibodies to CD14, CD19 (1 in 10 dilution) and MHC class II (1 in 20 dilution) were added to the cell suspension for at least 30 minutes at 4°C. The cell mixture was mixed gently by rotation. It was important to add the monoclonal antibodies in sufficient amounts to ensure optimal blocking of the antigen binding sites on the cell surface (see below).

After 30 minutes the cells were collected by centrifugation at 800g for 5 minutes. The supernatants were discarded and the cells washed twice with cold HBBS to remove all unbound antibody. Cells were resuspended at 1 x 10^7 cells/ml and a known volume of washed Dynabeads added.

Dynabeads are supplied as a suspension containing sodium azide. The volume of Dynabeads to be used in the purification process was transferred to a suitable washing tube which was placed in a magnet for one minute: the liquid was carefully pipetted off. The tube was removed from the magnet and an ample amount of HBBS was added to resuspend the Dynabeads. This whole process was repeated twice.

2.9.3 Calculation for volume of Dynabeads used.

At least 4 Dynabeads per estimated target cell ensures optimal separation.

Let the volume of heterogenous cell suspension, at 1 x 10^7 cells/ml, be **X** ml.

Percentage of targeted cells to be removed: 30% (0.3), ie. mononuclear phagocytes/monocytes/macrophages.

Bead density: 4 x 10^8 cells/ml.

So, the volume of Dynabeads to be used:

$$\frac{X \times (1 \times 10^7) \times 0.3 \times 4}{4 \times 10^8} = X \times 0.03\text{ml}.$$

2.9.4 Cell separation.

The calculated volume of Dynabeads was incubated with the cell suspension for at least 30 minutes at 4 °C. The cells were gently rotated during the incubation period. The sample tube was then placed onto a magnet for 5-10 minutes to collect the Dynabeads with cells attached. The supernatants were carefully pipetted off and the cells were washed twice with HBBS at 300g for 5 minutes. The purified T cells were then resuspended at the required density.

This procedure led to preparations of 93-96% purified T lymphocytes, as judged by flow cytometry described in section 2.10.

2.10 Flow cytometric measurement of T lymphocyte purity.

Flow cytometry can be used to identify cells by surface antigen expression. Monoclonal antibodies against the cell surface markers CD3, CD4, CD14, CD19 and MHC class II were used.

PBMC and purified T lymphocytes were tested in parallel. "Cells" will refer to both the heterogeneous cell suspension of PBMC and purified T lymphocytes.

Cells were washed twice with HBBS and resuspended at 0.5-1 x 10^7 cells/ml in HBBS supplemented with 5% FCS and 10% rabbit serum. The staining procedure was done at 4°C to minimize internalisation of cell membrane proteins.

90µl of the cell suspension was placed into each sample well of a 96 well round bottomed micotitre plate. PBMC and T lymphocytes were placed in separate plates to prevent cross-contamination of cells between wells.

10µl of a 1 in 20 dilution of monoclonal antibody was added to the wells (first layer antibody). Control samples were supplemented with HBBS. The plates were then incubated at 4°C. After 1 hour the cells were washed by centrifugation at 300g for 3 minutes with 200µl HBBS. The supernatants were discarded and the cell pellet was resuspended over a vortex. Cells were washed twice with HBBS in this way.

100µl of the second layer antibody, FITC-labelled (rabbit) anti-mouse IgG (1 in 20 dilution), was added to each well and the plates were incubated in the dark at 4°C for

1 hour. Samples were washed twice with 200µl HBBS before being finally resuspended in 200µl 4% paraformaldehyde.

The cells were analysed on a Becton Dickinson FACscan flow cytometer. 1000 events were collected for each sample. A gate was present to eliminate any dead cells from the sample. Markers were created on frequency histograms of the samples stained with the second layer antibody to define non-specific staining with the FITC-labelled antibody. These markers were then superimposed onto the frequency histograms of the samples stained with the monoclonal antibodies (first layer) and the percentage of PBMC and purified T lymphocytes expressing each surface marker was calculated.

Figures 2.4 and 2.5 show a flow cytometric dot plot of PBMC and purified T lymphocytes respectively. Lymphocytes were first identified by their size and granularity and gated as shown in Figure 2.4. Each dot represents one cell. The monocyte population (referred to as population B in Figure 2.4) is virtually absent in a dot plot from a sample which has been purified using Dynabeads (discussed in section 2.9). The cells were better characterised by their membrane antigens as described above. The most useful marker for T cells is CD3, part of the T cell antigen receptor. Population A from a PBMC sample contained 79% CD3+, 22% CD14+ and 28% MHC class II+ cells, which was defined as the lymphocyte population. After purification of a mixed cell population, isolated from human blood (Figure 2.5), the gated population of cells (defined as the lymphocyte population A) contained more than 93% CD3+ cells. The number of monocytes, identified by the CD14 cell surface marker, was reduced by 50%.

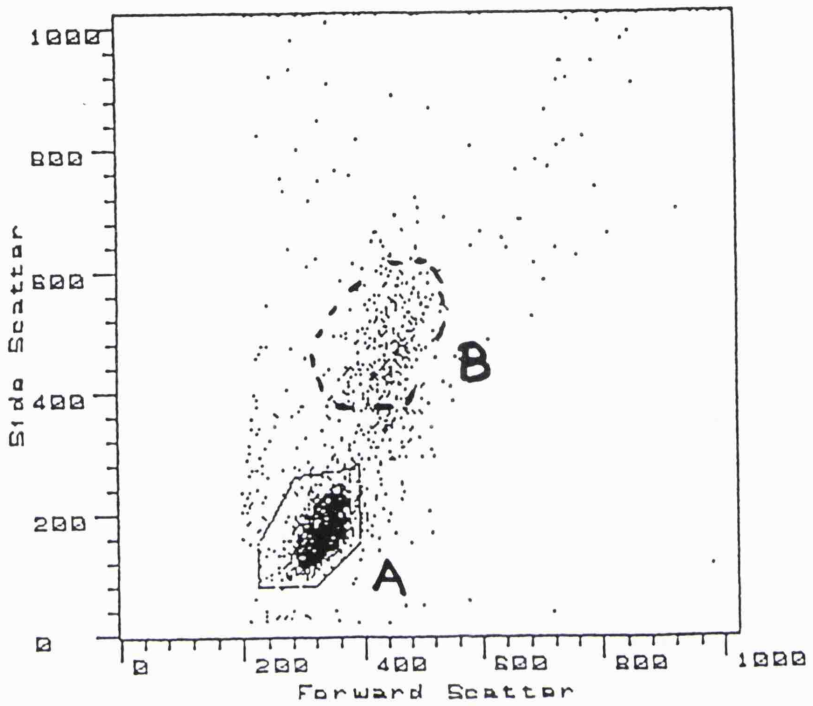


Figure 2.4

A Flow cytometric dot plot of PBMC displaying side scatter of light against forward scatter.

The cells labeled **A** are lymphocytes and the population contains predominantly T lymphocytes. Population **B** represents monocytes. Cell surface markers were used to identify the cells in population A. The cells in population A were: 79.21 ± 6.10 CD3+; 60.10 ± 1.19 CD4+; 36.10 ± 4.21 CD8+; 22.50 ± 12.22 CD14+; 28.10 ± 3.01 MHC class II+. Results are expressed as a percentage of the total population sample from three separate experiments.

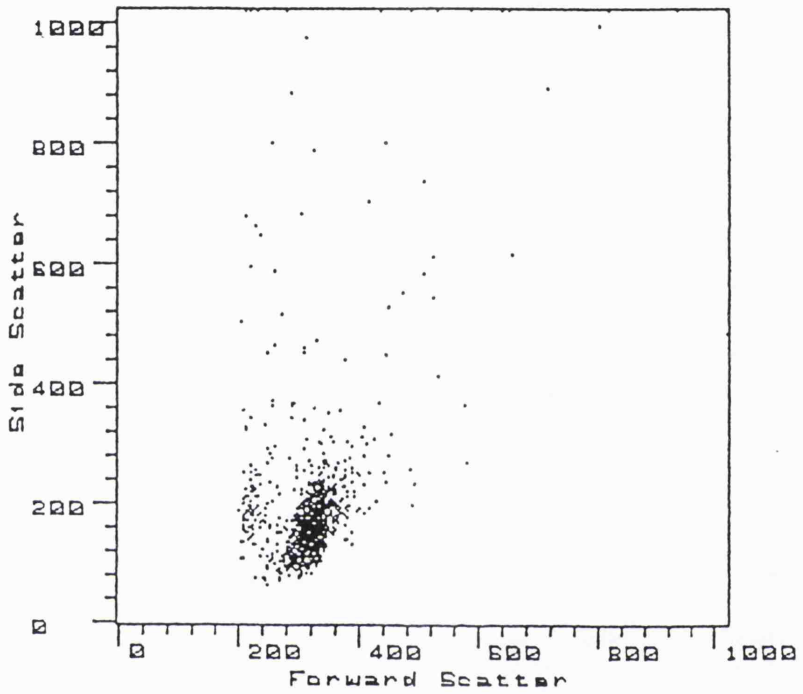


Figure 2.5

A flow cytometric dot plot of purified T lymphocytes displaying side scatter of light against forward scatter.

The cells were identified using cell surface markers as: 93.60 ± 2.31 CD3+; 64.11 ± 1.01 CD4+; 42.50 ± CD8+; 10.52 ± 1.17 CD14+; 10.00 ± 0.33 MHC class II+. Results are expressed as a percentage of the total population sample from three separate experiments.

2.11 Flow cytometric assay of ROS formation using DCF.

The assay measures the intracellular formation of oxidative products. Using a flow cytometer to gate separate cell populations allows the analysis of the fluorescence derived from monocytes and lymphocytes to be monitored separately. These two cell populations can be separated on the basis of their forward angle light scatter and their side angle light scatter (Figure 2.4).

2'7'-dichlorofluorescein diacetate (DCFH-DA) is a stable non fluorescent, non polar compound which can diffuse through cell membranes. Once inside the cell, cytosolic esterases cleave the acetyl groups from the molecule, producing a non-fluorescent reduced form of the compound, 2'7'- dichlorofluorescein (DCFH).

$$\underset{\substack{\text{non fluorescent}\\ \text{non polar}}}{\text{DCFH-DA}} \xrightarrow{\text{cytosolic esterases}} \underset{\substack{\text{non fluorescent}\\ \text{polar}}}{\text{DCFH}} + \text{ROS} \Rightarrow \underset{\text{fluorescent}}{\text{DCF}}$$

In the presence of ROS, most probably hydrogen peroxide (Roth and Valet, 1990) [Figure 2.6], DCFH is modified to a highly fluorescent oxidized form, DCF, which can be monitored on the flow cytometer.

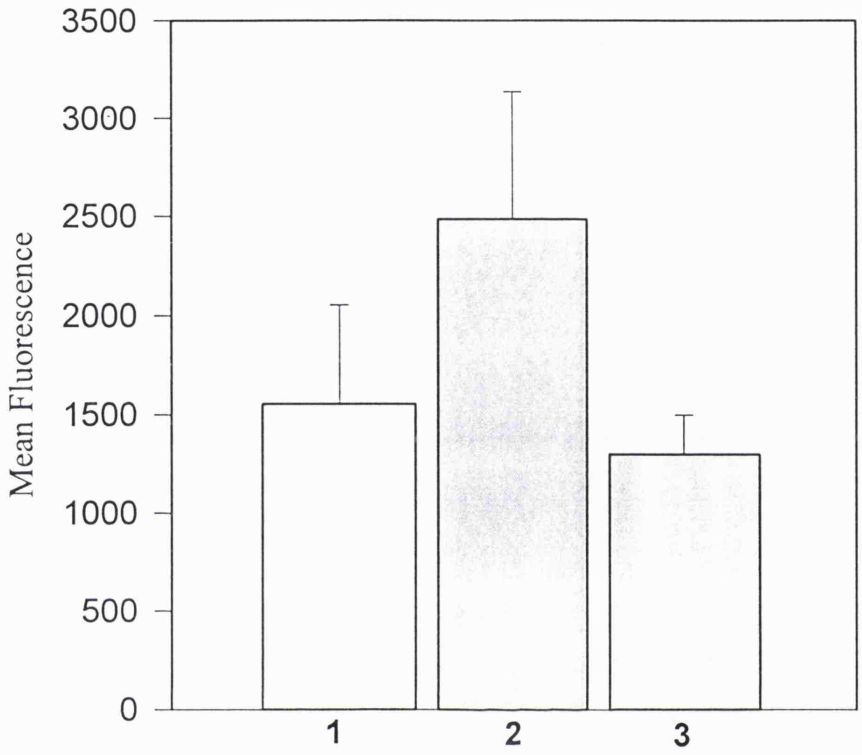


Figure 2.6
The effect of hydrogen peroxide on DCF fluorescence of PBL.
The effect of hydrogen peroxide on ROS production by the lymphocyte population of PBMC. Readings were taken every minute after the addition of: (**1**) hydrogen peroxide, 10nM; (**2**) hydrogen peroxide, 100nM. Results show the mean fluorescence data obtained after 5 minutes of stimulation. (**3**) Cells were also treated with the iron chelator desferrioxamine, 10μM, 30 minutes before the addition of hydrogen peroxide, 100nM. The results show the mean and s.e.m. from three separate experiments.

Protocol.

DCFH was stored in desiccant at -20°C and dissolved in ethanol as needed. All drugs were prepared on the day of the experiment in PBS. Cells were washed twice with PBS containing 0.4mM calcium and 0.8mM magnesium, before being resuspended at 4×10^7 cells/ml in PBS without magnesium and calcium. Calcium was finally excluded to minimise coagulation and "clumping" of cells. Cell suspensions were kept on ice prior to loading with the dye.

50µl of the cell suspension was diluted with 450µl of PBS (with magnesium and calcium). Where appropriate 250µl of the antioxidant was added instead of a volume of 250µl PBS for 15 minutes before the addition of DCFH-DA (final concentration 10µM).

Cells were incubated with the dye for 15 minutes in a 37°C water bath before the addition of stimulants. 250µl of stimulants were added to the cells in 12ml polystyrene centrifuge tubes. Where a combination of stimulants was used, 125µl of each was added simultaneously. Thus the final volume in each tube was 1ml. Readings were taken immediately after the addition of stimulants and every 5 minutes thereafter for 40 minutes. 5000 individual data points were collected for each sample point on a Becton Dickinson FACscan flow cytometer. The lymphocyte population was selected by gating during analysis, and the mean level of fluorescence (FL_1) was calculated and converted to linear units by the equation;

$$FL_1 = 10^{(\text{mean gate channel} / 256)}$$

Control samples, supplemented with equivalent volumes of PBS rather than stimulants were prepared in parallel. Mean fluorescence data was collected at each time point from cultures stimulated with drugs and from control samples. Mean fluorescence readings from the control samples were subtracted from the readings from drug-treated samples at each time point. This was an important procedure for two reasons: (i) to allow for any spontaneous oxidation of the dye; (ii) to exclude any small ROS fluxes continuously produced by cells as part of the electron transfer

chains in respiration. Hence all changes in the DCF fluorescence could be directly related to the effects of the stimulant. Where antioxidants were used, the mean fluorescence of a sample treated with antioxidant in the absence of stimulants was subtracted. The mean fluorescence of a sample treated with antioxidant was often lower than one which was supplemented with PBS. This could be due to the action of the antioxidant on any small amounts of ROS produced in respiration.

2.12 Coating of purified T cells with rabbit anti-mouse IgG.

Overview.

T cell activation through antigen recognition can be mimicked with monoclonal antibodies to the CD3-T cell antigen receptor. However, an important parameter in T cell receptor-mediated activation is the aggregation of several T cell receptors in close proximity. It is thought that immobilization of anti-CD3 through monocyte Fc receptors favours such aggregation (cross-linking) and is therefore an important requirement for T cell stimulation. It was important to determine if cross-linking was important for ROS production by T cells.

A suspension of purified T cells does not contain any monocytes and hence no monocyte Fc receptors. In order to cross-link anti-CD3 without monocyte Fc receptors, the experiments were carried out in the presence of a "cross-linking" rabbit anti-mouse immunoglobulin. The rabbit anti-mouse immunoglobulin provides a frame for binding and immobilizing the Fc part of anti-T cell monoclonal antibodies. Three different protocols were used to cross-link CD3 molecules.

Protocol 1.

500µl anti-CD3, 1.6µg/ml, was mixed with an equal volume of rabbit anti-mouse immunoglobulin, 3/50 dilution, at 4°C. (Final concentration of rabbit anti-mouse immunoglobulin and anti-CD3 after the addition of all other compounds was 1/100 dilution and 0.1µg/ml respectively). Control samples contained 500µl of PBS instead of anti-CD3 or rabbit anti-mouse immunoglobulin. The mixture of "pre-formed

complexes" was added to the purified T cell suspension after the cells had been loaded with the fluorescent dye, as described in section 2.11.

Protocol 2.

Step1- 1ml anti-CD3, 0.2μg/ml, was added to 1ml of a purified T cell suspension at 8 x 10^6 cells/ml.

Step 2- The cell suspension was incubated for 15 minutes at 4°C.

Step 3- Cells were washed with HBBS and resuspended at 8 x 10^6 cells/ml.

Step 4- 125μl of rabbit anti-mouse immunoglobulin, 1/25 dilution, was added to 500μl of the cell suspension (final concentration of rabbit anti-mouse IgG was 1/100).

Step 5- Cell samples were incubated for a further 15 minutes and then loaded with the dye.

Protocol 3.

The third protocol was the same as above but step 3 was excluded.

2.13 Experimental strategy.

Experiments were performed to measure ROS fluxes in T cells during the activation process and correlate the measured fluxes with subsequent cell proliferation or gene activation. Three lymphocyte functions were studied and compared:

(i) Early lymphocyte responses to stimulation (G_1 in the cell cycle) were measured by assaying IL-2 release into the culture supernatant from the cells.

(ii) The later S phase responses were measured by determining the amount of [^{3}H]-thymidine incorporation into DNA.

(iii) ROS production was measured using a free radical sensitive dye 2'7'-dichlorofluorescein (DCFH-DA) on a fluorescence activated cell sorter (FACS), hence allowing the measurement of intracellular ROS at a single cell level.

The relationship between ROS and all responses was further examined using ROS scavengers with differing modes of action. The concentration of antioxidant needed to promote 50% inhibition of IL-2 release, proliferation and ROS generation were determined.

Experimental results are expressed as the mean value obtained from n experiments. All points have vertical error bars which represent the standard error of the mean (s.e.m.). Where the error bars are not visible they are contained within the limits of the symbol. Statistical differences were calculated using the unpaired Student's t test and 2-way analysis of variance (ANOVA). Where * appears next to a symbol it indicates that that particular point is significantly different ($P<0.05$) to its corresponding control sample. # denotes that that particular point is not significantly different ($p>0.05$) from the control sample, but that all other points on the graph are. Hill plots were used to determine the concentration of antioxidant needed to inhibit the response by 50% (IC_{50}).

For DCF fluorescent measurements, at least 5000 events were acquired on the flow cytometer for each sample.

CHAPTER 3

ROLE OF REACTIVE OXYGEN SPECIES IN THE T CELL ACTIVATION PATHWAYS STIMULATED BY A PHORBOL ESTER AND CALCIUM IONOPHORE.

Introduction.

The experiments performed in this chapter used a phorbol ester, phorbol 1,2 myristate acetate (PMA), in combination with a calcium ionophore, A23187. This combination of stimuli has been extensively used as a model of the physiological mode of T cell activation by MHC-restricted antigen presentation (Nishizuka, 1986). The increase in intracellular calcium, by a calcium ionophore, and protein kinase C activation, by a phorbol ester, can bypass the requirements for receptor-induced signals at the onset of lymphocyte activation. However, how these two pathways act in concert for T cell activation and the nature of the biochemical signals involved is incompletely understood.

The effect of varying the concentrations of the calcium ionophore and phorbol ester on IL-2 release and cell proliferation was examined. The protocol for these experiments briefly was that the cells were incubated with a range of PMA and A23187 concentrations. Radioactive thymidine was added after a 32 hour incubation. After a further 16 hours cells were harvested, thymidine incorporation measured, and IL-2 content in the supernatants determined, as described more fully in chapter 2.

The synergistic effect between PMA and A23187 for IL-2 release and proliferation was also tested for free radical production using the fluorescent dye 2'7-dichlorofluorescein.

To establish further the role of ROS in the synergism between PMA and A23187, the sensitivity of the three lympocyte functions (IL-2 release, proliferation and ROS generation) to antioxidants was tested. The two antioxidants used were the iron chelator desferrioxamine and ascorbic acid.

3.1 Effect of A23187 on PMA-induced proliferation.

The results in Figure 3.1 show that increasing concentrations of A23187 from 32nM to 125nM, potentiated the response of suboptimal concentrations of PMA in inducing a proliferative response in PBMC.

PMA alone, over the range 0.125-2nM, had little effect on thymidine incorporation. A low A23187 concentration, 32nM, produced no significant change ($p>0.05$) in the PMA-induced cell proliferation with all PMA concentrations tested. However, larger concentrations of A23187, 62nM and 125nM, potentiated PMA-induced thymidine incorporation by more than 85% at a PMA concentration of 0.5nM.

3.2 Effect of A23187 on PMA-induced IL-2 release.

Figure 3.2 demonstrates that increasing concentrations of A23187 potentiated the effect of suboptimal concentrations of PMA in inducing IL-2 release.

From cell population to cell population, IL-2 release varies and so the data have been normalised by expressing IL-2 release as a percentage of the release caused by PMA stimulation alone, ie. as a percentage of the corresponding response to PMA in the absence of A23187.

As for proliferation, PMA had no significant effect on IL-2 release alone. The potentiation by A23187 (32-125nM) of PMA-induced IL-2 release was significant ($p<0.05$) only with PMA concentrations greater than 0.125nM. A23187, 62nM and 125nM, increased IL-2 release by more than 90% in combination with a PMA concentration between 0.5 to 2nM.

From these data, a concentration of PMA, 1nM, and A23187, 125nM, was chosen for subsequent experiments.

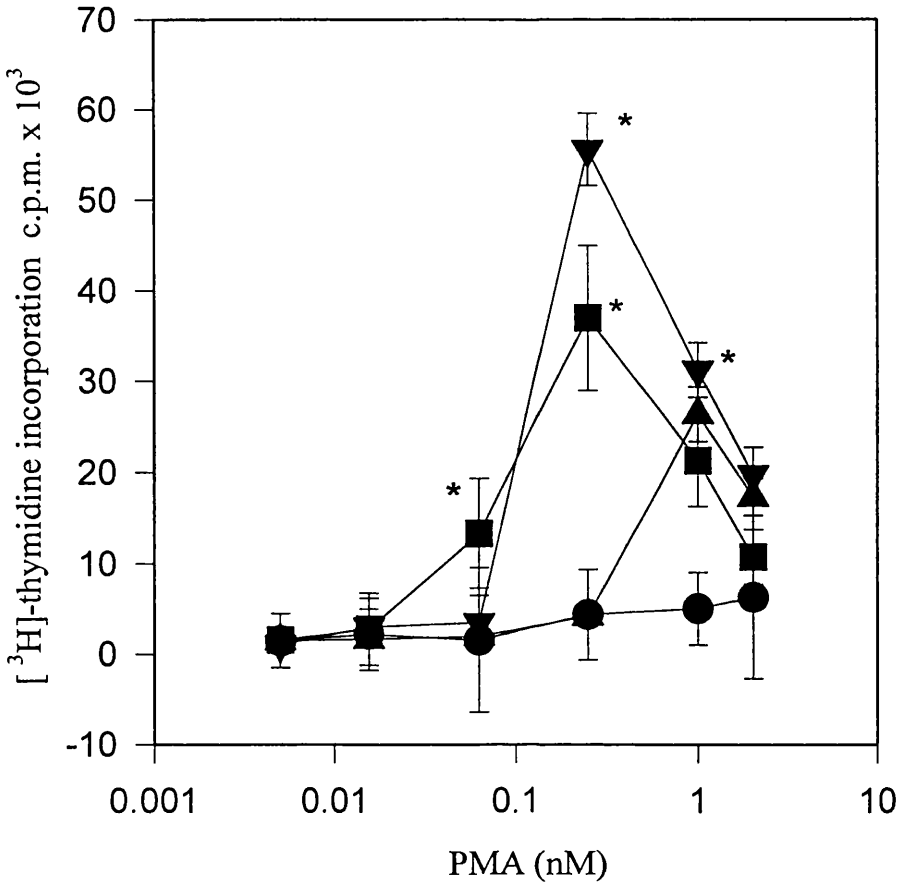


Figure 3.1
The influence of A23187 on the PMA-induced thymidine incorporation.
Effect of A23187 on proliferation stimulated by a range of PMA concentrations. Cells were treated with: ● PMA alone; ▲ PMA + A23187, 32nM; ■ PMA + A23187, 62.5nM; ▼ PMA + A23187, 125nM. PMA and A23187 were added at the initiation of culture and a pulse of [^{3}H]-thymidine was added for the final 16 hours of the 48 hour incubation. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) to the corresponding point in the absence of A23187 using the Student's unpaired t test.

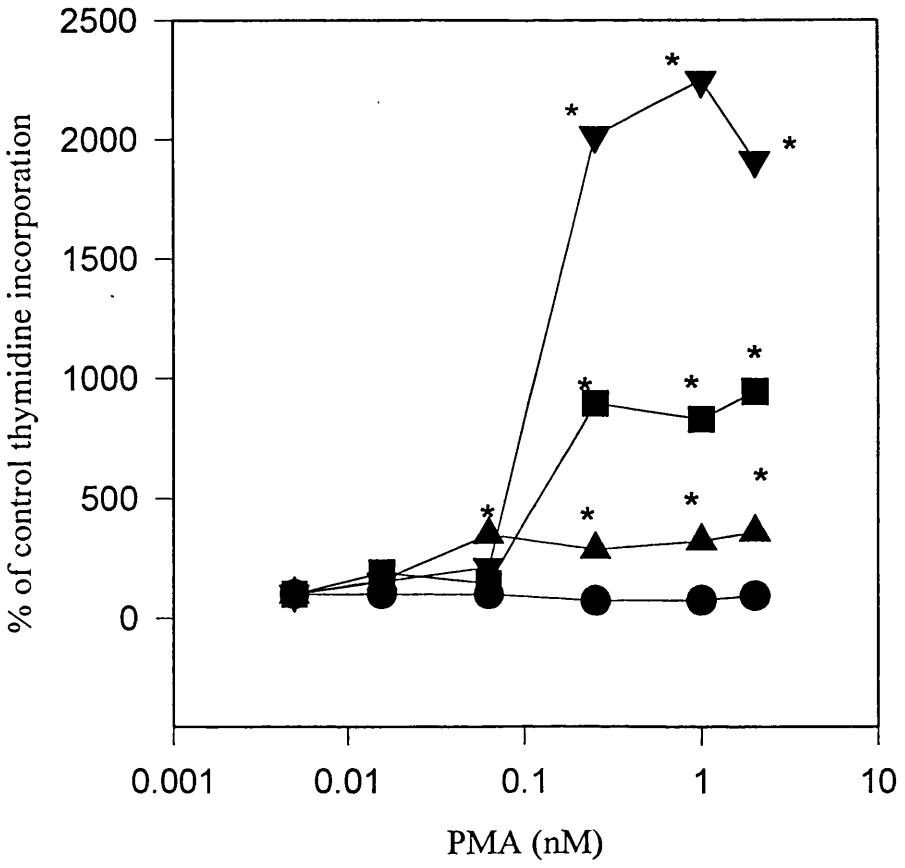


Figure 3.2
The influence of A23187 on PMA-induced IL-2 release.
Effect of A23187 on IL-2 secretion stimulated by a range of PMA concentrations. IL-2 was measured in the supernatants 48 hours after stimulation with: ● PMA alone; ▲ PMA + A23187, 32nM; ■ PMA + A23187, 62.5nM; ▼ PMA + A23187, 125nM. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. Results are expressed as % of the control [^{3}H]-thymidine incorporation of CTLL, which was equivalent to approximately 0.48pg/ml IL-2.
* indicates that a point is significantly different ($p<0.05$) to the corresponding point in the absence of A23187 using the Student's unpaired t test.

3.3 Effect of A23187 on PMA-induced free radical production.

To establish whether ROS may be involved in the synergistic action between PMA and A23187, the two drugs were tested alone and in combination for free radical generation. ROS production was assessed by an increase in the fluorescence of the free radical-sensitive dye, 2'7'-dichlorofluorescein (10μM).

Figure 3.3 shows that PMA, 1nM, alone generates a small fluorescence signal: the maximum mean fluorescence change was 495 units. This ROS signal increased over 30-40 minutes, before reaching a plateau. However, the combination of PMA and ionophore gave a much larger increase in signal. As for proliferation (Figure 3.1) and IL-2 release (Figure 3.2), maximal potentiation in response was achieved with a combination of PMA, 1nM, plus A23187, 125nM. Following an initial delay of 20 minutes after the addition of stimuli, A23187, 125nM, 85nM and 62.5nM potentiated the PMA-induced ROS signal by 85, 75 and 55% respectively. The increase in fluorescence started with a delay of 10 to 20 minutes and was then linear over the next 20-25 minutes, finally reaching a plateau at 40 minutes.

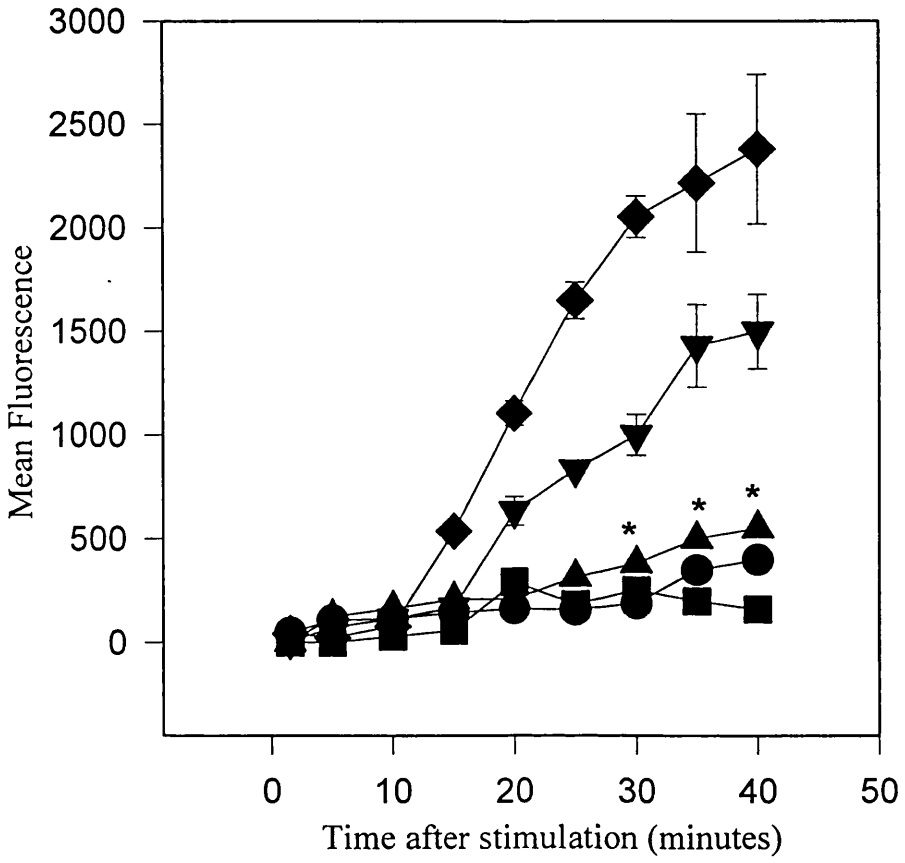


Figure 3.3
The influence of A23187 on the DCF fluorescence from PMA-stimulated PBMC. Cells were loaded with DCFH-DA, 10μM, for 15 minutes and then stimulated with: ● PMA,1nM alone; ■ PMA,1nM + A23187,32nM; ▲ PMA,1nM + A23187, 62.5nM; ▼ PMA, 1nM + A23187, 85nM; ◆ PMA,1nM + A23187,125nM. Mean fluorescence data from parallel cultures in the absence of PMA and A23187 were subtracted at each time point . The data are the mean ± s.e.m. from three separate experiments. * indicates that a point is significantly different ($p<0.05$) to the corresponding point in the absence of A23187 using the Student's unpaired t test. 2-way ANOVAs were used to statistically analyse the data. The p values obtained for PMA only vs PMA + A23187, 85nM and for PMA only vs PMA + A23187, 125nM were 0.0015 and 0.0001 respectively.

Effect of antioxidants on the proliferative response stimulated by PMA in synergy with A23187.

Much of the argument linking ROS production to T cell activation rests on the effects of free radical scavengers or inhibitors and antioxidants on the T cell response.
The two antioxidants used in this section were desferrioxamine and ascorbic acid. Desferrioxamine is an iron chelator and reduces ROS levels primarily through its removal of iron needed for the Haber Weiss and Fenton reactions. Ascorbic acid acts a proton donor and, unlike desferrioxamine, exerts its action on preexisting ROS. Both antioxidants were present throughout the 48 hour culture period.

3.4 Effect of desferrioxamine on proliferation.

Figure 3.4 shows that desferrioxamine produced a concentration-dependent inhibition of cell proliferation. This inhibition was observed over a range of desferrioxamine concentrations from 0.024 to 50μM. The concentration of desferrioxamine needed to promote 50% inhibition of the control thymidine incorporation (in the absence of desferrioxamine), was 0.45 ± 0.21μM. Greater than 75% inhibition of proliferation was achieved at concentrations of desferrioxamine greater than 1μM.

3.5 Effect of ascorbic acid on proliferation.

The results in Figure 3.5 show that ascorbic acid at concentrations greater than 1.25mM inhibited PMA/A23187 induced proliferation dose-dependently. The concentration of ascorbic acid needed to inhibit cell proliferation by 50% of its control response (PMA + A23187, in the absence of ascorbic acid) was 2.97 ± 0.13mM. Thus, ascorbic acid was found to be about 6000 times less potent in this respect than desferrioxamine.
These concentrations of desferrioxamine and ascorbic acid did not affect CTLL proliferation in the presence of a submaximal concentration of human r-IL-2. Hence, their inhibitory effect is not associated with cytotoxicity or interference with the assay for IL-2.

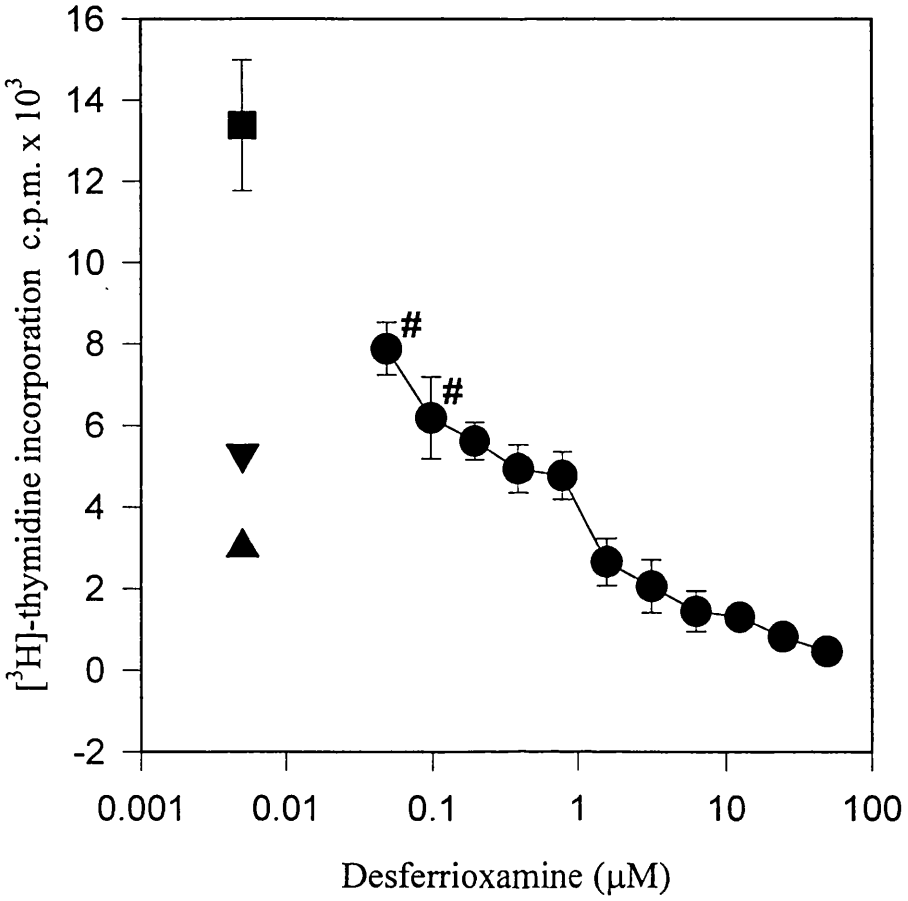


Figure 3.4
The effect of desferrioxamine on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of desferrioxamine on proliferation of PBMC stimulated with PMA,1nM, plus A23187,125nM. Cells were incubated with desferrioxamine at the concentrations shown. PMA and A23187 were added simultaneously. A pulse of [^{3}H]-thymidine was added for the final 16 hours of the 48 hour incubation. ▼ PMA,1nM alone; ▲ A23187,125nM alone ; ■ PMA,1nM + A23187, 125nM ; ● PMA, 1nM + A23187, 125nM + desferrioxamine. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. # indicates that a point is not significantly different ($p>0.05$) from control values in the absence of desferrioxamine, using the Student's t-test. The IC_{50} of desferrioxamine was 0.45 ± 0.21μM.

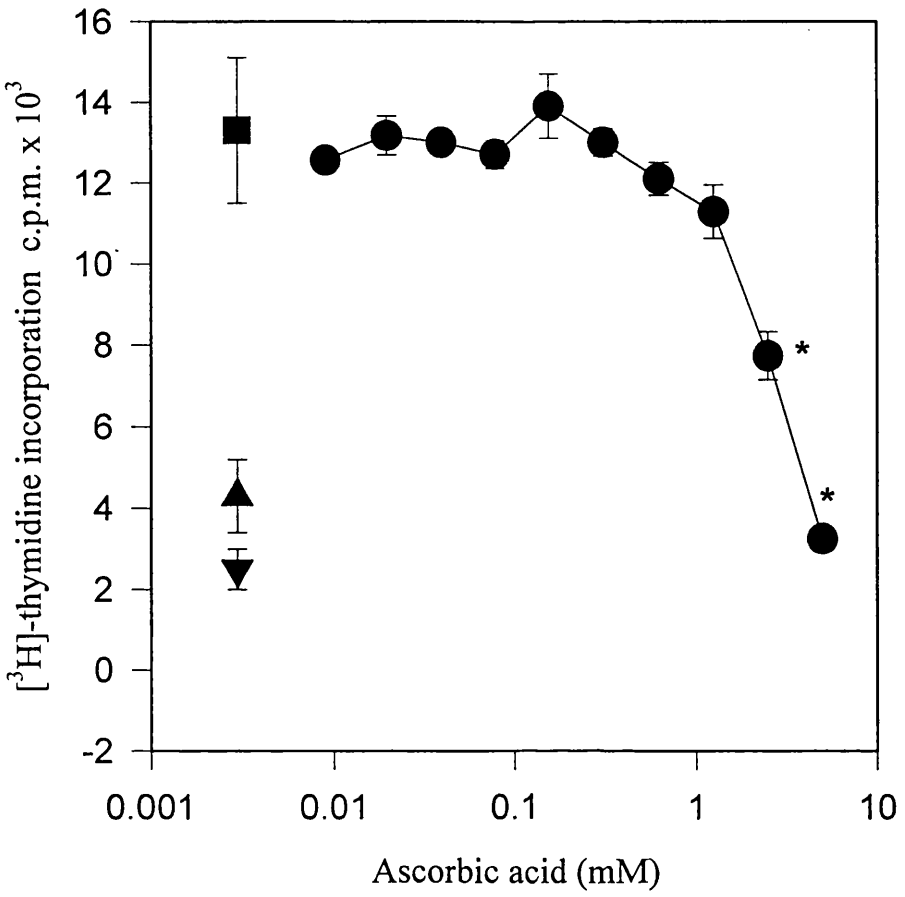


Figure 3.5
The effect of ascorbic acid on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of ascorbic acid on proliferation of PBMC stimulated with PMA,1nM plus A23187, 125nM. PBMC were incubated with ascorbic acid at the concentrations shown, PMA and A23187 were added simultaneously and the cells were incubated for 48 hours prior to the measurement of thymidine incorporation. ▲ PMA,1nM alone; ▼ A23187,125nM alone (ie. in the absence of ascorbic acid) ; ■ PMA,1nM + A23187, 125nM; ● PMA,1nM + A23187,125nM ÷ ascorbic acid. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($P<0.05$) from control values in the absence of ascorbic acid. The IC_{50} of ascorbic acid was 2.97 ± 0.13 mM.

Effect of antioxidants on IL-2 release stimulated by PMA in synergy with A23187.

Unlike the actions of desferrioxamine and ascorbic acid on the proliferative response stimulated by PMA, 1nM, plus A23187, 125nM, both antioxidants had no significant effect on IL-2 release.

The IL-2 released from antioxidant treated samples is expressed as a percentage of the IL-2 released from cells stimulated by PMA plus A23187, in the absence of antioxidant.

3.6 Effect of desferrioxamine on IL-2 release.

Figure 3.6 shows that desferrioxamine, 0.024-50μM, produced no significant change in the IL-2 release by PBMC.

3.7 Effect of ascorbic acid on IL-2 release.

Figure 3.7 shows that ascorbic acid also produced no significant change ($p>0.05$) in the IL-2 release over the entire range of ascorbic acid concentrations tested; 0.002-5mM.

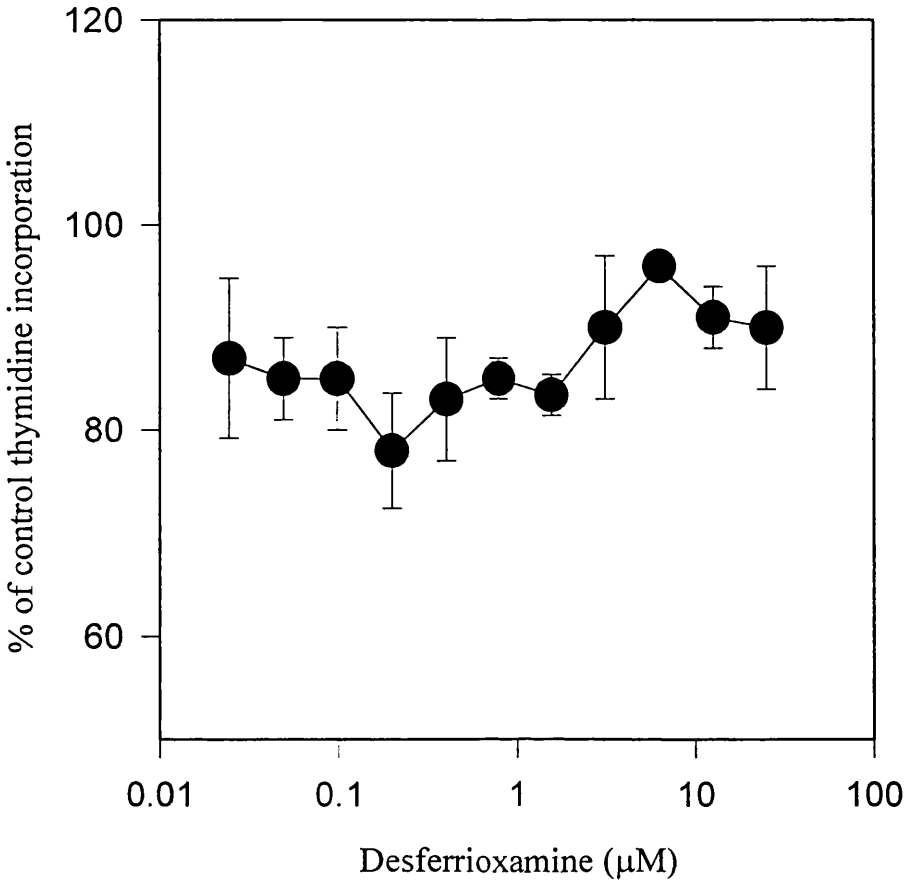


Figure 3.6
The effect of desferrioxamine on IL-2 release by PBMC.
The concentration-response relationship for the effect of desferrioxamine on IL-2 production by PBMC stimulated with PMA,1nM plus A23187,125nM. PBMC were incubated with desferrioxamine at the concentrations shown. PMA and A23187 were added simultaneously and the cells were incubated for 48 hours prior to the collection of supernatants for IL-2 assay. Background levels of IL-2 release were less than 900cpm, equivalent to less than 0.001ng/ml. Results are expressed as % of the thymidine incorporation in the control sample (ie. PMA + A23187 in the absence of ascorbic acid) which was 22000cpm, equivalent to approximately 2.5ng/ml IL-2. PMA and A23187 gave counts of less than 2000 (< 0.005ng/ml IL-2). Each point represents the mean ± s.e.m of triplicate determinations from three independent experiments.

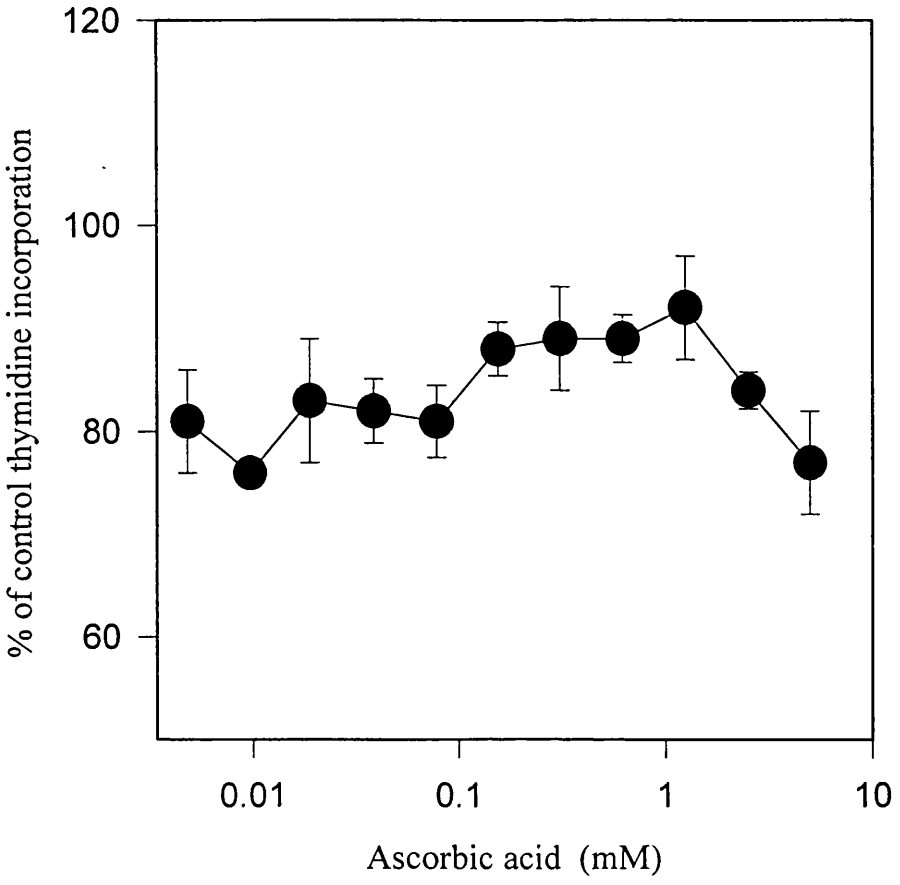


Figure 3.7
The effect of ascorbic acid on IL-2 release by PBMC.
The concentration-response relationship for the effect of ascorbic acid on IL-2 production by PBMC stimulated with PMA, 1nM plus A23187, 125nM. PBMC were incubated with ascorbic acid at the concentrations shown. PMA and A23187 were added simultaneously and cells were cultured for 48 hours prior to the collection of supernatants for IL-2 assay. Results are expressed as the % of the thymidine incorporation in the control samples (PMA + A23187 in the absence of ascorbic acid), equivalent to 2.5ng/ml IL-2. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments.

Effect of antioxidants on ROS formation stimulated by PMA plus A23187.

The antioxidants, desferrioxamine and ascorbic acid, were added to the cells 15 minutes prior to the addition of the dye. The cells were then incubated for a further 15 minutes before stimulation with PMA, 1nM, plus A23187, 125nM. Mean fluorescence readings were subtracted from parallel cultures incubated with the antioxidant, but not stiimulated with PMA+A23187, at each time point.

3.8 Effect of desferrioxamine on ROS formation.

Figure 3.8 shows that desferrioxamine blocks ROS formation in a dose-dependent manner. The inhibition was significant for desferrioxamine 0.01μM, 0.1μM and 10μM ($p<0.05$) at the end of the 40 minute period studied.

From analysis of the inhibitory dose-response curves for ROS generation and proliferation, the concentration of desferrioxamine required for 50% inhibition of proliferation is higher (0.45μM) than that required to inhibit ROS formation by a similar degree (0.01μM).

3.9 Effect of ascorbic acid on ROS formation.

Figure 3.9 shows that ascorbic acid blocks ROS formation dose-dependently.

Concentrations of 0.5μM, 31.25μM and 1.25mM all produced a significant reduction ($p<0.05$) in DCF fluorescence (as determined at the end of the 40 minute period). Ascorbic acid, 1.25mM, completely abrogated the ROS signal over the entire 40 minute period.

Again, the concentration of ascorbic acid needed for 50% inhibition of proliferation (2.97mM) was higher than that required to inhibit ROS generation by a similar degree (0.5μM).

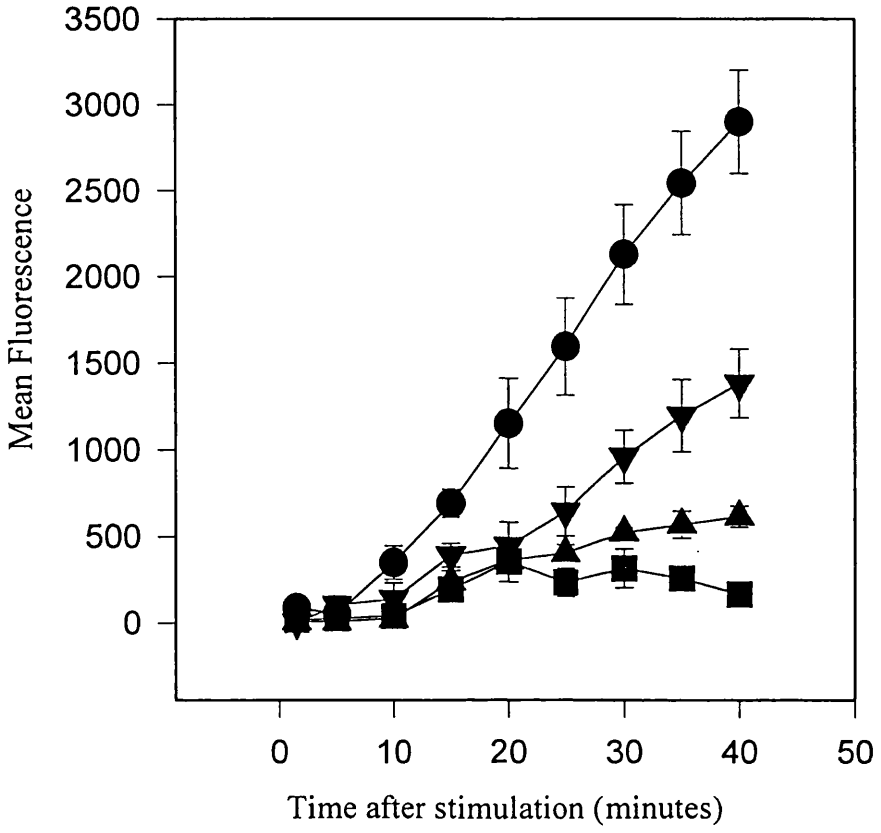


Figure 3.8

The effect of desferrioxamine on the DCF fluorescence of PBL.

The concentration-response relationship for the effect of desferrioxamine on ROS production by the lymphocyte population from PBMC, stimulated with PMA,1nM plus A23187,125nM. Desferrioxamine or medium was added to the cells 15 minutes before the addition of DCFH-DA,10μM. The cells were incubated for a further 15 minutes before stimulation with PMA + A23187. Mean fluorescence readings were taken every 5 minutes for 40 minutes and subtracted from parallel cultures in the absence of PMA and A23187. Cells were treated with: ● no desferrioxamine; ▼ 0.01μM desferrioxamine; ▲ 0.1μM desferrioxamine; ■ 10μM desferrioxamine. The data are the mean ± s.e.m. from three separate experiments. 2-way ANOVAs were used to statistically analyse the data. The p values obtained for no desferrioxamine vs 10μM, 0.1μM and 0.01μM desferrioxamine, following a 20 minute delay, were all less than 0.05.

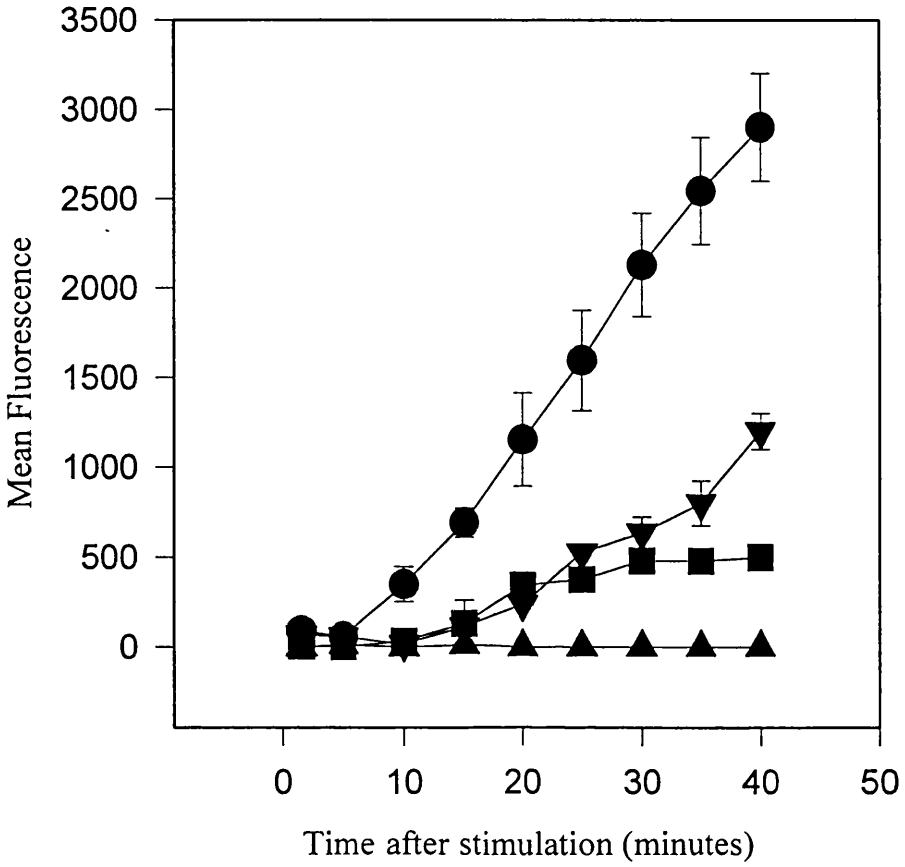


Figure 3.9
The effect of ascorbic acid on the DCF fluorescence of PBL.
The concentration-response relationship for the effect of ascorbic acid on ROS production by the lymphocyte population from PBMC, stimulated with PMA, 1nM plus A23187,125nM. Ascorbic acid or medium was added to the cells 30 minutes before stimulation. Mean fluorescence readings were taken every 5 minutes and subtracted from parallel cultures in the absence of PMA + A23187. Cells were treated with: ● no ascorbic acid; ▼ 0.5μM ascorbic acid; ■ 31.25μM ascorbic acid; ▲ 1.25mM ascorbic acid. The data are the mean ± s.e.m from three separate experiments. 2-way ANOVAs were used to analyse the data following a delay of 20 minutes. The p values obtained for no ascorbic acid vs 0.5μM, 31.25μM and 1.25mM ascorbic acid were all less than 0.05.

3.10 Effect of catalase on the ROS signal produced by PMA plus A23187.

Catalase is a large protein (240KD), unable to cross cell membranes and enter the cytoplasm. Hence, it acts on ROS produced outside the cells.

The concentration of catalase was selected from previous studies that showed catalase, at concentrations greater than 1000U/ml, was effective in inhibiting DCF fluorescence induced by PMA-activated neutrophils (Rabesandratana *et al.*, 1992).

Figure 3.10 illustrates the effect of catalase, 1500U/ml, on the response to PMA plus A23187 on free radical production. Catalase had no significant effect on the DCF fluorescence, implying that the ROS signal being measured was formed intracellulary. A higher concentration of catalase, 3000U/ml was also without effect on free radical production (not shown).

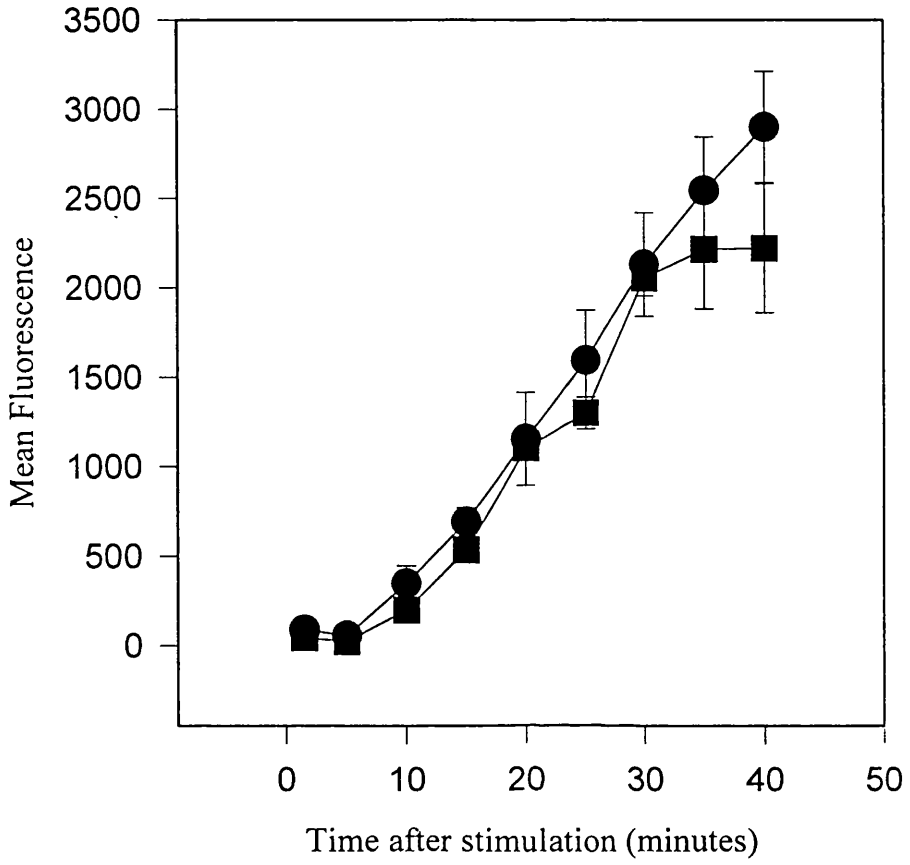


Figure 3.10
The effect of catalase on the DCF fluorescence of PBL stimulated with PMA and A23187.
Cells were pretreated with either catalase, 1500 U/ml (■), or medium (●), for 15 minutes prior to the addition of DCFH-DA,10μM. Cells were incubated for a further 15 minutes before the addition of PMA,1nM plus A23187,125nM. Fluorescence data was collected every 5 minutes over a 40 minute period. Mean fluorescence data from parallel cultures in the absence of PMA and A23187 were subtracted at each time point.

Discussion.

The aim of the experiments in this chapter was to examine the role of ROS in the T cell activation pathways stimulated by a phorbol ester, PMA, to activate PKC and a calcium ionophore, A23187, to promote a calcium signal inside the cell.

Originally it was thought that ROS generation was confined to certain cells of the immune system, namely phagocytes. However, strong evidence implicating oxidative phenomena in T cells (Sekkat *et al.,* 1987) has questioned the importance of ROS as intracellular "messengers" in the signalling cascade. The question of whether there are specific molecular "targets" for oxidative signalling, or whether ROS influence signalling pathways through indirect means, was approached in these experiments and those in subsequent chapters.

Generation of ROS was measured using the free radical sensitive dye, 2'7'-dichlorofluoroscein (DCF). In experiments conducted with PBMC, the cell populations were separated on the basis of the forward angle light scatter and side angle light scatter: different gates could be used to identify lymphocytes, neutrophils and monocytes. Therefore, DCF fluorescence readings (ROS signal) throughout the experiments were measured specifically in the lymphocyte population.

DCF was used in conjunction with desferrioxamine and ascorbic acid (two antioxidants whose common property is their ability to prevent formation of, or, inactivate ROS), in order to evaluate the importance of ROS as signalling molecules. By comparing the sensitivity of ROS fluxes, the release of IL-2 and T cell proliferation to these antioxidant compounds, it was possible to analyse whether the redox state of the cell, or whether oxygen radicals were playing the determinant role in regulation of T cell activation (a similar strategy was also used in subsequent experimental work).

How ROS function as regulatory factors remains speculative. A number of studies performed by Schreck and co-workers have provided evidence to suggest that activity of the transcription factor, NF-κB, is controlled by a redox-dependent mechanism. First, hydrogen peroxide induced NF-κB activity in Jurkat cells (Schreck *et al.,* 1991). Thereafter, the chemically different antioxidants, NAC and pyrrolidine

dithiocarbamates inhibited NF-κB activation in T cells which had been activated by several unrelated agonists such as TNF-α and IL-1 (Schreck *et al.,* 1992). The mechanism by which NF-κB is activated is not completely clear, but evidence for the involvement of protein kinases has been obtained from studies which showed that the protein kinase C activator, PMA, induces NF-κB activation (Sen *et al.,* 1986). This was an important reason for choosing PMA as a model to induce T cell activation in experiments throughout this thesis.

NF-κB plays an important part in the synthesis of IL-2 and expression of the IL-2 receptor (section 1.2.5). IL-2 gene expression is rapidly induced following mitogenic T cell stimulation and is essential for proliferation of these cells. The mechanisms involved in the regulation of expression of the IL-2 gene have been extensively studied and therefore provide a useful framework for the identification of molecular targets of oxidative signalling during the initial events of T cell activation.

Treatment of PBMC with A23187 increases the membrane permeability to calcium, resulting in an increased cytosolic calcium concentration. The site of action of the phorbol ester, PMA, is thought to be protein kinase C, a calcium and phospholipid-dependent kinase. PMA is structurally related to 1,2-diacylglycerol, the physiological activator of protein kinase C. The results presented in Figures 3.1 and 3.2 are in agreement with previous studies (Truneh *et al.,* 1985; Nishizuka, 1986); neither A23187 nor PMA were effective alone, but a combination of these agents could act in concert and enhance IL-2 release and T cell proliferation. Although the synergistic effect between calcium ionophores and phorbol esters is well documented, less evidence is available about the nature of the biochemical pathways mediating this effect.

The concentrations of both compounds appeared to be crucial to the response. It has previously been demonstrated that a concentration of A23187 greater than 500nM causes irreversible phosphorylation of some proteins and more than 10nM PMA might act to disrupt the membrane in addition to its action on protein kinase C (PKC) (Kumagai *et al.,* 1987). Maximal responses were observed over a narrow range of PMA and A23187 concentrations (0.5-1nM PMA and 62.5-125nM A23187). When

submaximal concentrations of both agents were used, this resulted in synergistic effects on proliferation and IL-2 release. These data imply that an intracellular calcium signal together with PKC activation both play a part in T cell activation. A concentration of 1nM PMA and 125nM A23187 was chosen for subsequent experiments using this activation model.

In order to test the theory that ROS may be involved in the synergistic effect of these two agents for proliferation and IL-2 release, PMA and A23187 were tested alone and in combination for free radical production (Figure 3.3). Activation of T cells in a mixed population of peripheral blood leukocytes with PMA, 1nM, induced an increased production of ROS. These data support previous findings that have showed T lymphocytes, in the presence of accessory cells, are capable of ROS generation (Whitacre *et al.*, 1992; Rabesandratana *et al.*, 1992).

The results showed that, as for proliferation and IL-2 release, increasing concentrations of A23187 synergised with PMA, 1nM, for free radical production. Accordingly, a concentration of 125nM A23187 with PMA, 1nM, gave a maximal response. These data suggest that T cell proliferation and IL-2 release induced by PMA in synergy with A23187 is accompanied by oxygen free radical production.

Neutrophil stimulation results in activation of the respiratory burst generating ROS, such as the superoxide anion radical and hydrogen peroxide. A simple explanation of ROS activity detected in T lymphocytes could be that hydrogen peroxide was generated in these phagocytic cells and then diffuses into the T cells. Catalase is a large antioxidant enzyme (40KD) which can not readily pass membranes (Beckman *et al.*, 1988). Rabesandratana *et al* (1992) showed that catalase at a concentration of 1000U/ml was effective at reducing the DCF fluorescence induced by PMA-stimulated neutrophils. The fact that catalase, at concentrations greater than or equal to 1500U/ml, produced no significant change in the DCF fluorescence induced by PMA in synergy with A23187 excludes this possibility, and confirms an intracellular site of ROS formation in T lymphocytes.

To examine the role of ROS in this activation model further, the effects of antioxidants on the T cell responses determined in Figures 3.1, 3.2 and 3.3 were

studied. The antioxidants used here were desferrioxamine, an iron chelator, and ascorbic acid. Although the iron chelator is being referred to as an antioxidant, it actually prevents the formation of radicals, primarily through its removal of the iron needed for the Haber Weiss and Fenton reactions. Ascorbic acid is a water soluble antioxidant which functions as a free radical scavenger by electron donation; hence it acts on pre-existing oxygen radical species.

The sensitivity of the proliferative response to desferrioxamine and ascorbic acid suggests that ROS may be involved in this response (Figures 3.4, 3.5). The results presented in this chapter showed that the concentration of antioxidant needed to inhibit proliferation was several orders of magnitude higher than that needed to inhibit DCF fluorescence, for example 30μM ascorbic acid blocked the ROS signal induced by PMA/A23187 by more than 80%, but had no significant effect on proliferation or IL-2 release. The discrepancy between the IC_{50} values of ascorbic acid and desferrioxamine for inhibition of proliferation and ROS formation suggests that the relationship between increased ROS production and subsequent events leading to T cell activation is not a simple one. An alternative explanation could be that these compounds have effects on proliferation which are not related to their antioxidant properties. This suggestion is supported by the results of previous findings: (i) iron is an important co-enzyme for certain enzymes, for example ribonucleotide reductase, hence the removal of iron would inhibit DNA synthesis (Carotenuto *et al.*, 1986).

(ii) Lipoxygenase enzymes, which are important in the metabolism of arachidonic acid, contain essential non-heme iron atoms. Desferrioxamine could bind these iron atoms and inhibit these enzymes (Fedyk *et al.*, 1993). Furthermore, arachidonic acid metabolism generates ROS as by-products (section 1.3.2), thus desferrioxamine could be indirectly inhibiting ROS formation through its action on these enzymes. (iii) Santos *et al.*, (1993) showed that iron may influence the expression of T lymphocyte surface markers (CD4 and CD2 down-regulation), which would ultimately affect T cell recognition, positioning and activation.

In contrast, desferrioxamine and ascorbic acid produced no significant change in the IL-2 release stimulated by a combination of PMA and A23187. Desferrioxamine and

ascorbic acid by themselves did not change the responsiveness of the CTLLs used to assay increased IL-2, or effect trypan blue exclusion of PBMC. This suggests that their action was not associated with cytotoxicity or interference with the assay of IL-2. These data (Figures 3.6, 3.7) confirm those found by Chaudri *et al* (1988).The proliferation of CTLL (cells already expressing IL-2 receptors) was not affected by any of the antioxidants used in this study, suggesting that these compounds act at a step before the expression of IL-2 receptors. These results are discordant with those using other models of activation, namely PMA plus anti-CD3 or anti-CD28 (see chapters 4, 5) which showed an antioxidant dose-dependent inhibition of IL-2 release. Hence, it is possible that the observations made by Chaudri *et al* could be related to the mode of stimulation used rather than the action of antioxidants. In addition they found that antioxidants inhibited the expression of IL-2 receptors and transferrin receptors. Expression of the IL-2 receptor precedes the expression of the transferrin receptor (Neckers *et al.,* 1983). Hence, it is possible that the inhibition of the IL-2 receptor is the primary event which leads to the subsequent decrease in transferrin receptor expression. Whether this inhibition occurs at the level of gene transcription or is a later event cannot be determined from this work. Regulation of the IL-2 receptor gene is less stringently controlled than the IL-2 gene. Indeed IL-2 binding can induce the expression of its own receptor. It is, therefore, not surprising that inhibition of IL-2 release would consequently inhibit the expression of the IL-2 receptor. The mode of stimulation used as the important factor, rather than the effect of the antioxidants, is further supported by other reports using PMA plus A23187 (Dornand *et al.,* 1989). The discrepancy found between using PMA plus A23187 and other activation models, could be related to the mode of action of the calcium ionophore. The action of A23187 is thought to be a result of at least two independent pathways; one IL-2 dependent, but the other IL-2 independent (Koretzky *et al.,* 1983). The interaction of any T cell with IL-2 is thought to eventually lead to calcium translocation. Because A23187 directly initiates a powerful calcium translocation, the calcium ionophore may eliminate the need for IL-2 interaction with T cells and so its action would be insensitive to the effects of an antioxidant.

A criticism of the above studies must be that the exact cellular site of action of these antioxidant compounds in inhibiting proliferation could not be determined. As discussed, it is possible that these compounds act by different mechanisms unrelated to their antioxidant properties. A wider range of antioxidant compounds with differing modes of action would help to resolve these data.

In summary, the experiments conducted in this chapter showed that potentiation of PMA-induced IL-2 release and proliferation by A23187, is paralleled by an increase in ROS generation. T cell proliferation was inhibited dose-dependently by desferrioxamine and ascorbic acid. IL-2 release was not sensitive to the effects of these compounds. However, it was shown that the concentration of antioxidant needed to inhibit proliferation by 50% was several orders of magnitude higher than that needed to inhibit DCF fluorescence reading.

In conclusion, the results provide evidence compatible with the hypothesis that cellular activation is tightly regulated by the redox status of the cell (Staal *et al.*,1994). However, it is unlikely that ROS act on specific "oxidative receptors". Indeed the relationship between ROS and T cell activation is more complex. Future studies directed towards the effects of ROS on intracellular glutathione levels and other aspects of signal transduction would help to clarify this.

CHAPTER 4.

ROLE OF REACTIVE OXYGEN SPECIES IN THE T CELL ACTIVATION PATHWAYS STIMULATED BY A PHORBOL ESTER AND ANTI-CD3: A MIXED CELL POPULATION.

Introduction.

The experiments presented in this chapter were designed to replace the calcium ionophore used in chapter 3 with a more physiological tool for T cell activation.

Anti-CD3 mAb $UCHT_1$ identifies the T cell antigen complex CD3 and is often used as a CD marker to identify T cells in a mixed cell population. Much evidence exists for an increase in intracellular calcium following anti-CD3 binding (O'Flynn *et al.*, 1986). In addition like the calcium ionophore, anti-CD3 can synergise with a phorbol ester for T cell activation (Weiss *et al.*, 1984).

First the synergism between anti-CD3 and phorbol 1,2 myristate acetate (PMA) for proliferation and IL-2 release was confirmed. Subsequently, anti-CD3 and PMA were tested for free radical production alone, and in combination at concentrations which were effective at enhancing thymidine incorporation and cytokine expression.

To examine the role of ROS in the activation pathways stimulated by anti-CD3 plus PMA further, the sensitivity of these pathways to antioxidants was studied. Three other antioxidants were used here, in addition to desferrioxamine and ascorbic acid; dimethylsulfoxide (DMSO), vitamin E (α-tocopherol) and N-acetylcysteine (NAC). These compounds are thought to exert their antioxidant actions by differing mechanisms. The concentrations of these compounds needed to inhibit proliferation, IL-2 release and ROS production, by a similar degree, was compared.

4.1 Effect of anti-CD3 on PMA-induced proliferation.

Figure 4.1 shows that anti-CD3, 0.1μg/ml, and PMA, 0.3nM, alone had little effect on thymidine incorporation by PBMC. However, a combination of anti-CD3 and PMA enhanced proliferation by 60% relative to either stimulus alone.

The fact that anti-CD3-induced proliferation was 80% greater than PMA-induced proliferation confirms the presence of accessory cells. Accessory cells are able to provide a "second signal" for T cell activation, the first being provided by anti-CD3.

4.2 Effect of anti-CD3 on PMA-induced IL-2 release.

As for proliferation, Figure 4.2 shows that anti-CD3, 0.1μg/ml, and PMA, 0.3nM, alone produced no significant IL-2 release. Results are expressed as the thymidine incorporated by CTLL supported by a 1:8 dilution of the culture supernatant. Anti-CD3 potentiated the effect of PMA in inducing IL-2 release; 93% increase in IL-2 release relative to either stimulus alone.

4.3 Effect of anti-CD3 on the PMA-induced free radical production.

Figure 4.3 shows that anti-CD3, 0.1μg/ml, alone had very little effect on ROS production; the maximum mean fluorescence change was 25 units. PMA, 0.3nM, alone produced a small, reproducible ROS signal; maximum mean fluorescence change of 450 units. However, as for PMA + A23187 (chapter 3), the combination of anti-CD3 and PMA gave a much larger and rapid increase in signal; maximum mean fluorescence change of 2300 units. Thus, there was a 80% increase in the DCF fluorescence relative to either stimulus alone.

The signal started with a delay of approximately 20 minutes, continued linear over the next 30-40 minutes, and did not reach a plateau during this time interval.

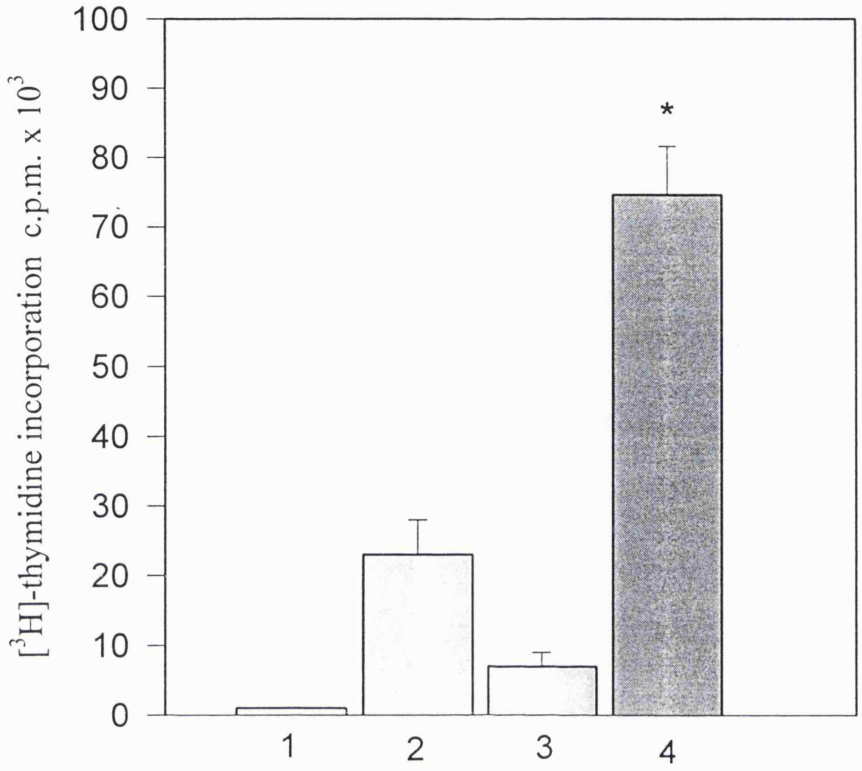


Figure 4.1
The influence of anti-CD3 on the PMA-induced thymidine incorporation.
Effect of anti-CD3, 0.1μg/ml, on proliferation of PBMC stimulated with PMA, 0.3nM. Cells were treated with: (1) medium alone; (2) anti-CD3, 0.1μg/ml alone; (3) PMA, 0.3nM alone; (4) PMA + anti-CD3. After 32 hours cells were pulsed with [^{3}H]-thymidine and incubated for a further 16 hours. Each bar represents the mean ± s.e.m. of triplicate determinations from three independent experiments.
* denotes that the data is significantly different ($p<0.05$) to either stimulus alone.

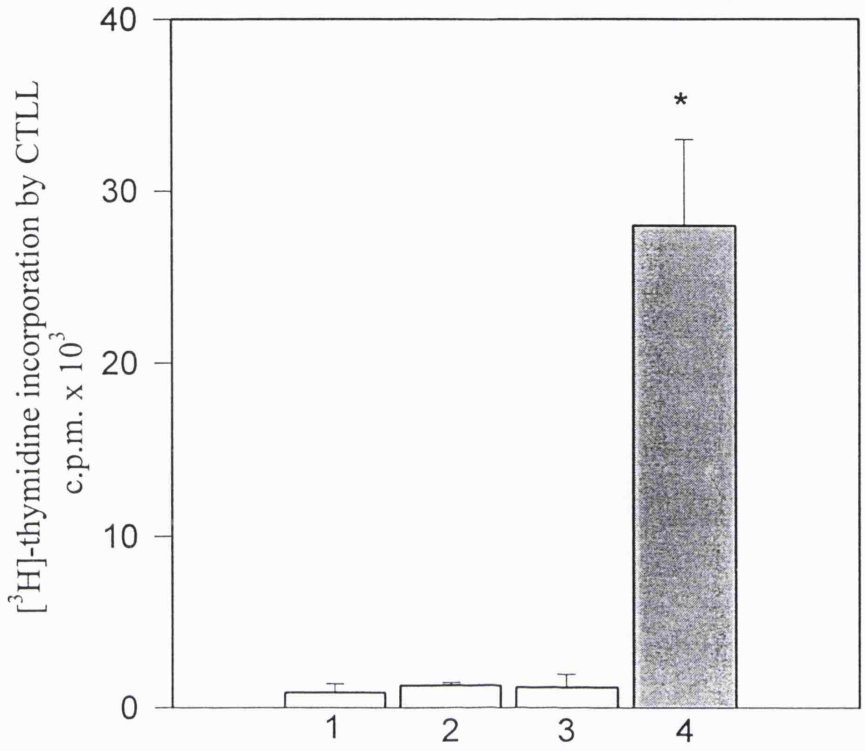


Figure 4.2
The influence of anti-CD3 on PMA-induced IL-2 release.
Effect of anti-CD3, 0.1μg/ml, on IL-2 secretion by PBMC stimulated with PMA, 0.3nM. Cells were treated with: (1) medium alone; (2) anti-CD3, 0.1μg/ml alone; (3) PMA, 0.3nM alone; (4) PMA + anti-CD3. After 48 hours of culture, cell supernatants were assayed for IL-2 using CTLL. Results show the thymidine incorporated by CTLL supported by a 1:8 dilution of the culture supernatant. Each bar represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that the data is significantly different ($p<0.05$) to either stimulus alone.

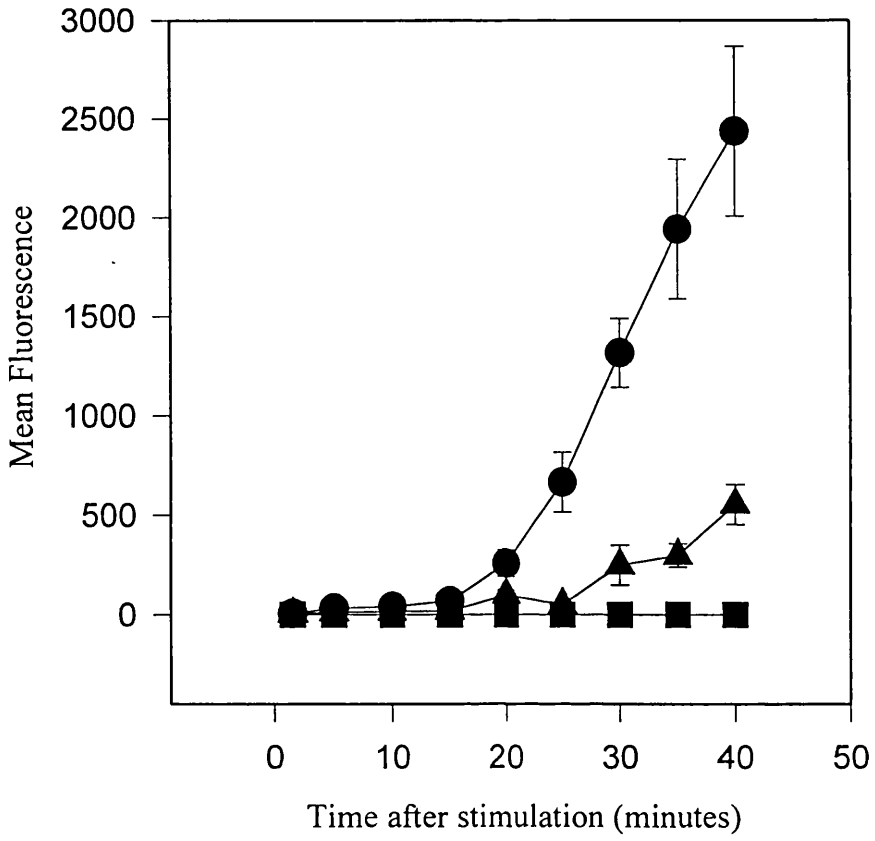


Figure 4.3
The influence of anti-CD3 on the DCF fluorescence from PMA-stimulated PBMC.
Effect of anti-CD3 on ROS production by the lymphocyte population of PBMC, stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were loaded with DCFH-DA, 10μM, 15 minutes before stimulation with: ▲ PMA, 0.3nM alone; ■ anti-CD3, 0.1μg/ml; ● PMA + anti-CD3. Fluorescence data were collected every 5 minutes, and subtracted from parallel cultures in the absence of PMA and anti-CD3 at each time point. The data are the mean ± s.e.m. from three separate experiments. 2-way ANOVAs were used to statistically analyse the data. The p value obtained for PMA + CD3 vs PMA alone is 0.0198.

4.4 Effect of desferrioxamine on the proliferative response stimulated by PMA in synergy with anti-CD3.

Figure 4.4 shows that deferrioxamine, 0.024-50μM, inhibited proliferation by PBMC dose-dependently. This effect ranged from about 15% below control (cells stimulated without desferrioxamine) at 0.1μM to about 85% below control at 50μM. The concentration of desferrioxamine needed to promote 50% inhibition of the control thymidine incorporation was 1.52 ± 0.13μM.

4.5 Effect of ascorbic acid on the proliferative response stimulated by PMA in synergy with anti-CD3.

Figure 4.5 shows that ascorbic acid dose-dependently inhibited PMA+anti-CD3-induced proliferation. This inhibitory effect was significant at concentrations of ascorbic acid between 0.3125-5mM. Ascorbic acid at concentrations less than 0.3125mM had effects which were not statistically different from those cells stimulated without ascorbic acid. The concentration of ascorbic acid that inhibited proliferation by 50% was 1.19 ± 0.46mM.

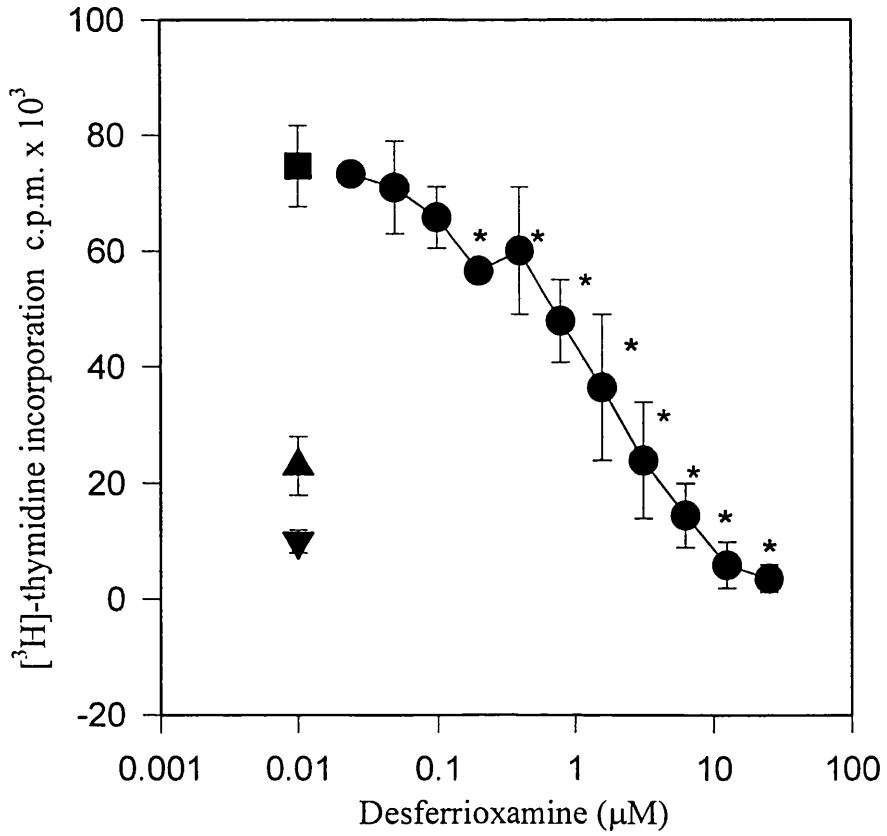


Figure 4.4
The effect of desferrioxamine on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of desferrioxamine on proliferation of PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were incubated with desferrioxamine at the concentrations shown. PMA and anti-CD3 were added simultaneously. After 32 hours cells were pulsed with [^{3}H]-thymidine and incubated for a further 16 hours. ▼ PMA, 0.3nM alone; ▲ anti-CD3, 0.1μg/ml alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + desferrioxamine. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of desferrioxamine, using the Student's t test. The IC_{50} of desferrioxamine was 1.52 ± 0.13μM.

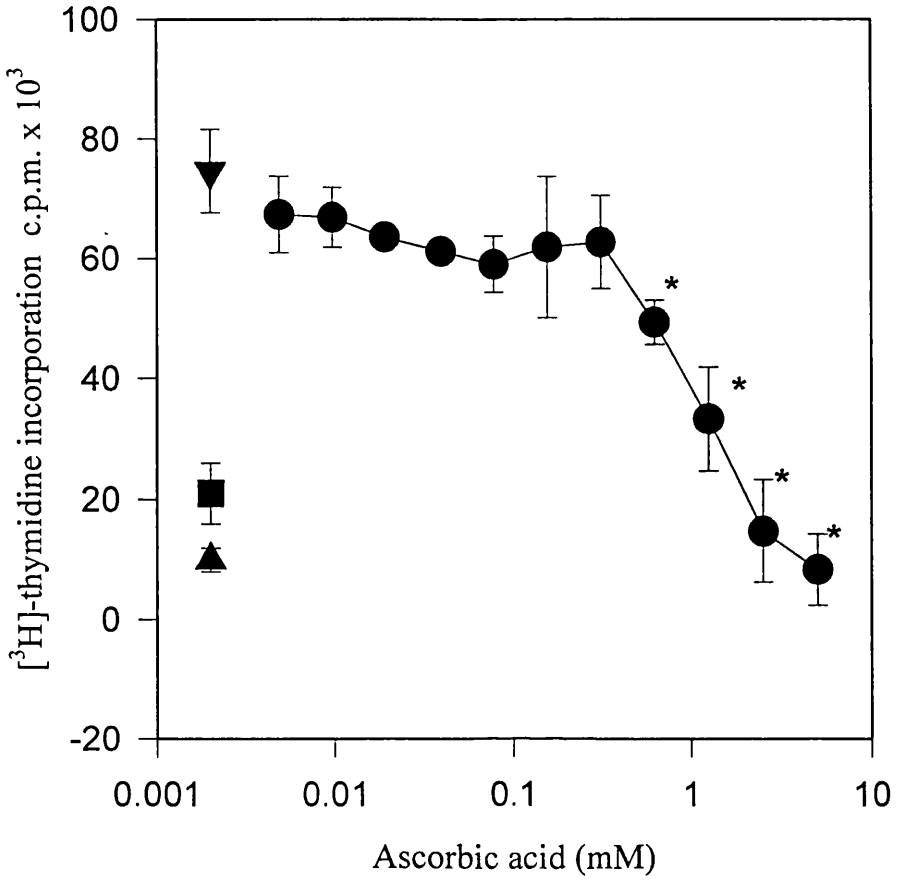


Figure 4.5
The effect of ascorbic acid on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of ascorbic acid on proliferation stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. PBMC were incubated with ascorbic acid at the concentrations shown. PMA and anti-CD3 were added simultaneously and a pulse of [^{3}H]-thymidine was added for the last 16 hours of the 48 hour incubation period. ▲ PMA, 0.3nM alone; ■ anti-CD3, 0.1μg/ml alone; ▼ PMA + anti-CD3; ● PMA + anti-CD3 + ascorbic acid. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of ascorbic acid, using the student's t test. The IC_{50} of ascorbic acid was 1.19 ± 0.46 mM.

All results for the effects of antioxidants on IL-2 release are expressed as a percentage of the thymidine incorporated by CTLL in the control sample. The control sample contained a 1:8 dilution of the culture supernatant from cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml, without antioxidant.

Cell viability and CTLL growth were unaffected by these antioxidant compounds at concentrations that inhibited PMA + anti-CD3-induced cell proliferation by more than 85%, unless otherwise stated; as such indicating that their inhibitory effect is not associated with cytotoxicity or interference with the assay for IL-2.

4.6 Effect of desferrioxamine on IL-2 release stimulated by PMA in synergy with anti-CD3.

Figure 4.6 shows that desferrioxamine, 0.024-50μM, caused a dose-dependent inhibition in the amount of IL-2 release stimulated by PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. This is in contrast to the action of desferrioxamine on IL-2 release stimulated by PMA + A23187 (Figure 3.6).

The inhibitory effect of desferrioxamine ranged from about 10% below control at 0.1μM to about 60% below control at 50μM. The concentration of desferrioxamine needed to promote 50% inhibition of the control IL-2 release was 86% larger than that needed to inhibit proliferation; 10.98 ± 1.92μM.

4.7 Effect of ascorbic acid on IL-2 release stimulated by PMA in synergy with anti-CD3.

Figure 4.7 shows that ascorbic acid at concentrations from 0.1 to 5mM caused a dose-dependent inhibition in the IL-2 release by PBMC. This effect ranged from about 5% below control at 0.1mM to almost 100% inhibition of IL-2 release at 5mM. The concentrations required to inhibit proliferation (1.19mM) and IL-2 release (1.03mM) by 50% below control were very similar.

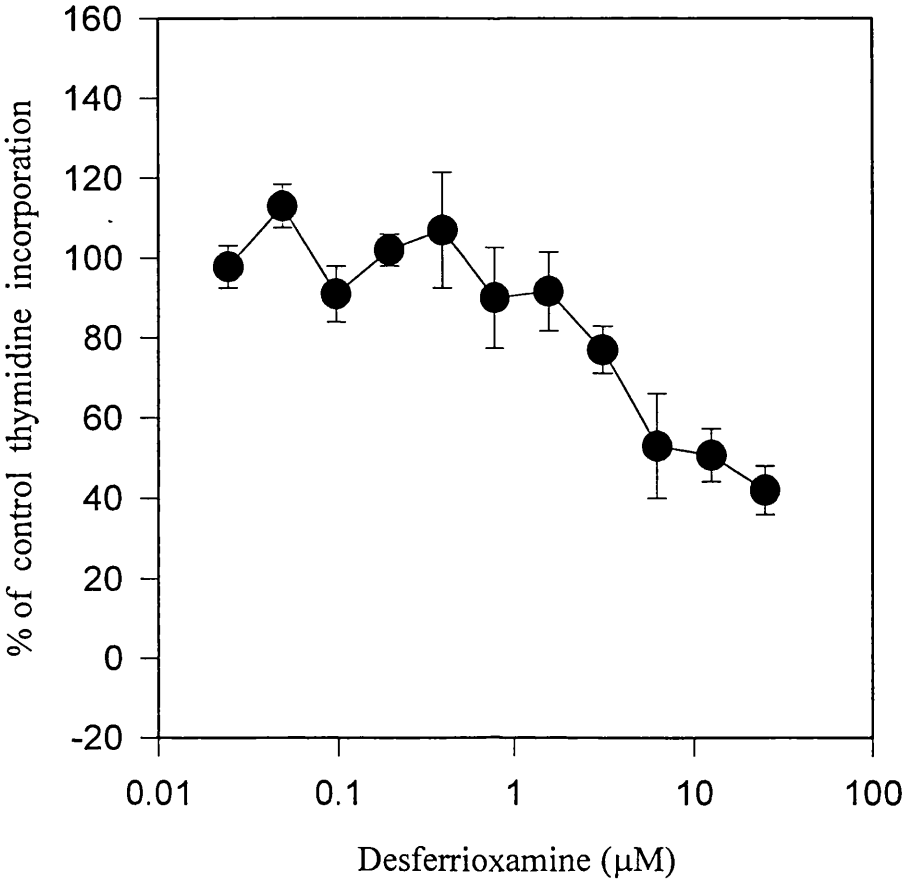


Figure 4.6
The effect of desferrioxamine on IL-2 release by PBMC.
The concentration-response relationship for the effect of desferrioxamine on IL-2 production by PBMC stimulated with PMA, 0.3nM plus anti-CD3, 0.1μg/ml. PBMC were incubated with desferrioxamine at the concentrations shown. PMA and anti-CD3 were added simultaneously and the cells were incubated for 48 hours prior to the collection of supernatants for IL-2 assay. Background levels of IL-2 release were less than 900 cpm, equivalent to less than 0.001ng/ml IL-2. Results are expressed as % of the control thymidine incorporation (ie. PMA + anti-CD3 in the absence of desferrioxamine) which was 21000 ± 6000 cpm and is equivalent to approximately 2.5ng/ml IL-2. PMA and anti-CD3 alone gave counts of less than 2000, equivalent to approximately 0.0039ng/ml IL-2. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. The IC_{50} of desferrioxamine was 10.98 ± 1.92 μM.

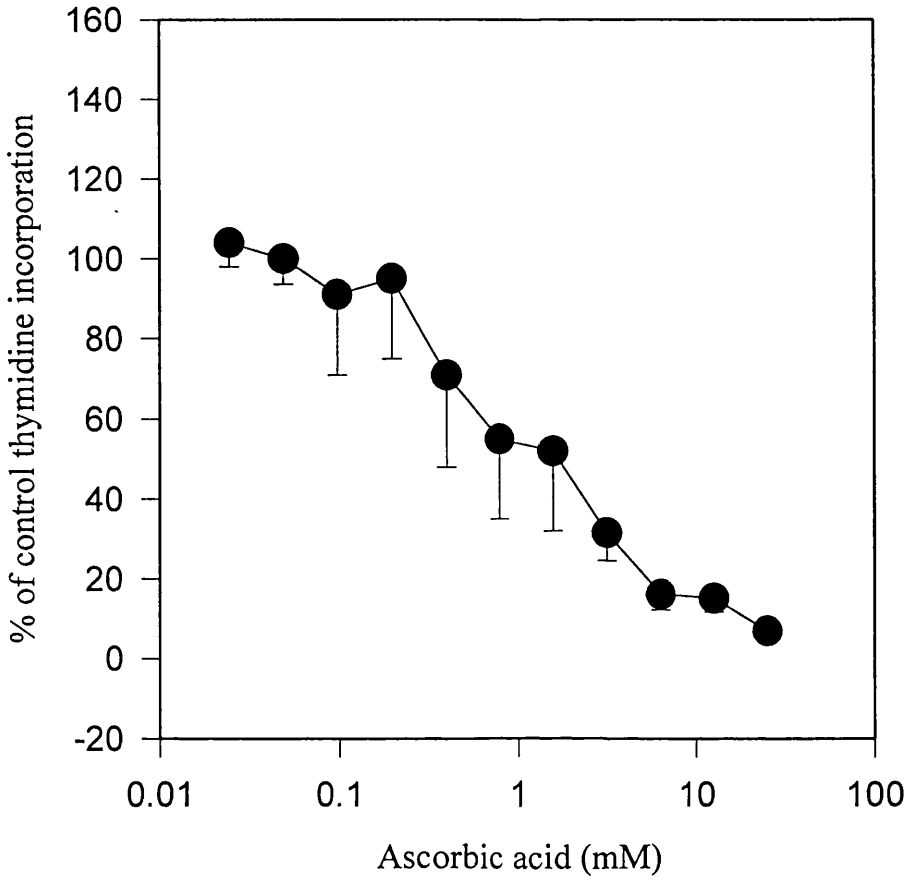


Figure 4.7
The effect of ascorbic acid on IL-2 release by PBMC.
The concentration-response relationship for the effect of ascorbic acid on IL-2 production by PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. PBMC were incubated with ascorbic acid at the concentrations shown. PMA and anti-CD3 were added simultaneously. Cells were cultured for 48 hours prior to the collection of supernatants for IL-2 assay. Results are expressed as the % of the thymidine incorporation in control samples (PMA + anti-CD3 in the absence of ascorbic acid), equivalent to 2.5ng/ml IL-2. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. The IC_{50} of ascorbic acid was 1.03 ± 0.13 mM.

4.8 Effect of desferrioxamine on free radical production stimulated by PMA plus anti-CD3.

Figure 4.8 shows that desferrioxamine was effective at inhibiting ROS production by the lymphocyte population from PBMC. This effect was dose-dependent. The DCF fluorescence of cells treated with 0.01 and 10μM desferrioxamine was reduced by 73 and 92% of the control sample respectively. The control samples were stimulated with anti-CD3, 0.1μg/ml, plus PMA, 0.3nM, without antioxidant.

From analysis of the inhibitory dose-response curves for ROS generation and proliferation, the concentration of desferrioxamine required to inhibit proliferation by 50% below control was about 99% larger in magnitude than that needed to inhibit free radical generation.

4.9 Effect of ascorbic acid on free radical production stimulated by PMA plus anti-CD3.

Figure 4.9 shows that ascorbic acid dose-dependently inhibited ROS generation induced by PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. At the end of the 40 minute period studied, cells treated with 0.5μM and 0.5mM ascorbic acid produced 35% and 96% reduced DCF fluorescence readings relative to the control readings respectively. The inhibition of ROS generation by ascorbic acid, 0.5mM, was statistically significant ($p<0.05$) over the entire 40 minute period.

As for desferrioxamine, the concentration of ascorbic acid needed to inhibit proliferation 50% below control was higher than that needed to inhibit ROS generation; almost 100% greater in magnitude.

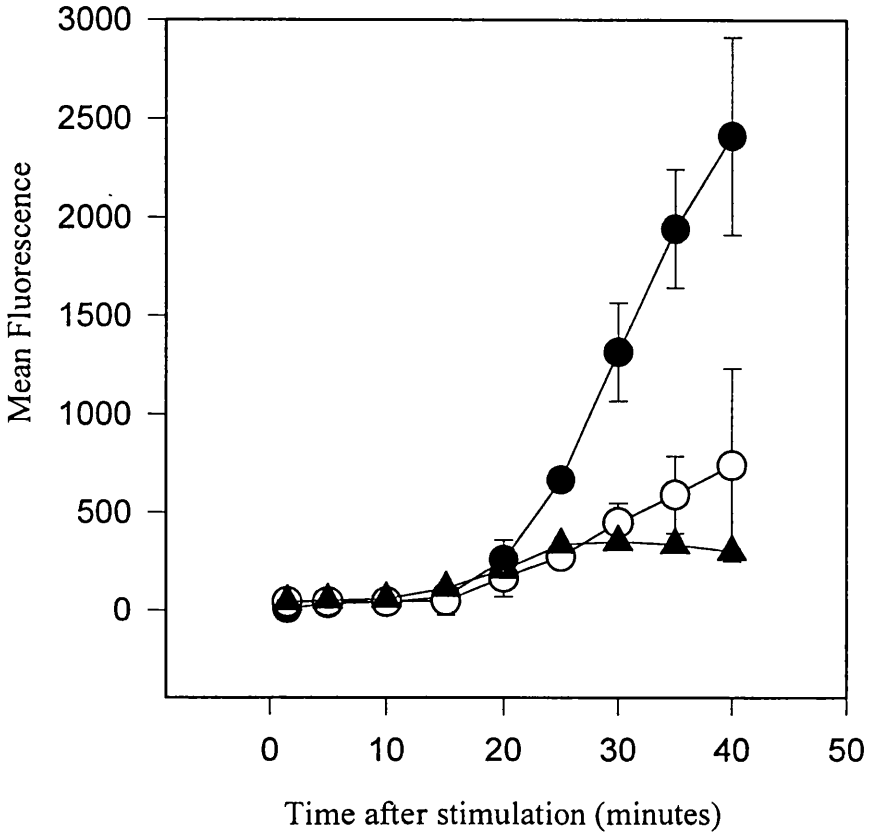


Figure 4.8
The effect of desferrioxamine on the DCF fluorescence of PBL.
The concentration-response relationship for effect of desferrioxamine on ROS production by the lymphocyte population from PBMC, stimulated with PMA, 0.3nM plus anti-CD3, 0.1μg/ml. Desferrioxamine or medium was added to the cells 30 minutes before stimulation. Mean fluorescence readings were taken every 5 minutes and subtracted from parallel cultures in the absence of PMA and anti-CD3. Cells were treated with: ● no desferrioxamine; ○ 0.01μM desferrioxamine; ▲ 10μM desferrioxamine. The data are the mean ± s.e.m. from three separate experiments.
2-way ANOVAs were used to statistically analyse the data. The p values obtained for no desferrioxamine vs 0.01μM and 10μM desferrioxamine, following a 20 minute delay, were 0.03 and 0.01 respectively.

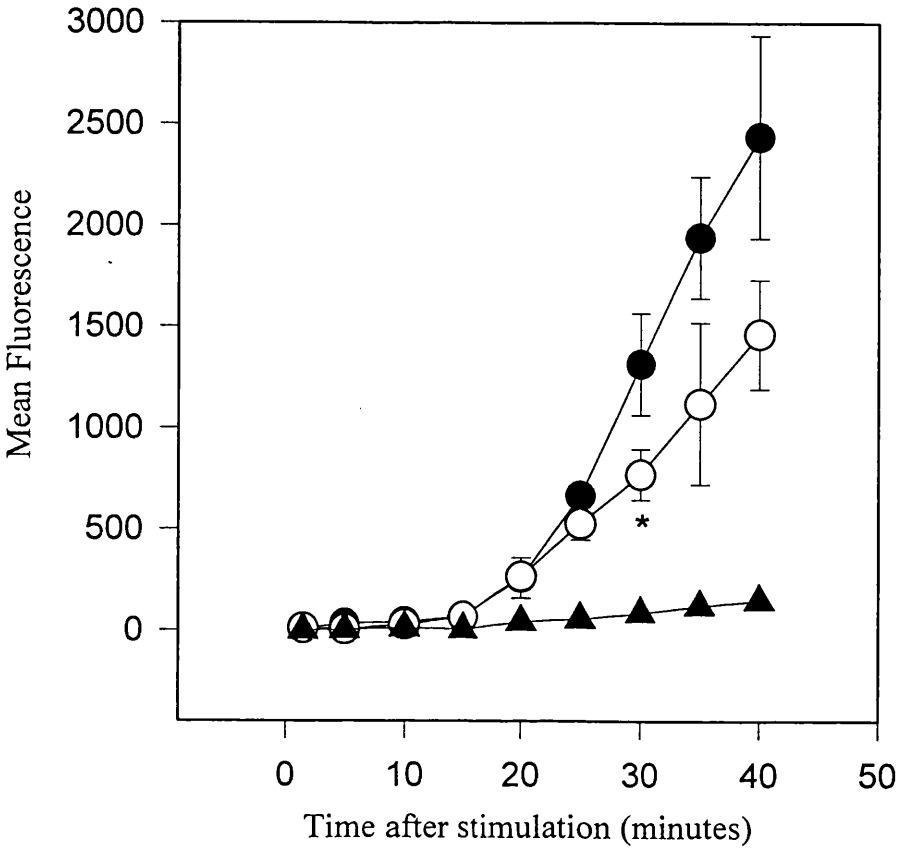


Figure 4.9
The effect of ascorbic acid on the DCF fluorescence of PBL.
The concentration-response relationship for the effect of ascorbic acid on ROS production by the lymphocyte population from PBMC, stimulated with PMA, 0.3nM, plus anti CD3, 0.1μg/ml. Ascorbic acid or medium was added to the cells 30 minutes prior to the addition of stimulus. Mean fluorescence readings were taken every 5 minutes over the 40 minute period. Cells were treated with: ● no ascorbic acid; ○ 0.5μM ascorbic acid; ▲ 0.5mM ascorbic acid. The data are the mean ± s.e.m. from three separate experiments. * indicates that a point is significantly different to that of the control, ie without ascorbic acid. 2- way ANOVAs were used to analyse the data. The p value obtained for no ascorbic acid vs 0.5mM ascorbic acid was 0.008.

To investigate further whether PMA acts in synergy with anti-CD3 through ROS-mediated signalling, three other antioxidants were used; dimethyl sulfoxide (DMSO), vitamin E and N-acetylcysteine (NAC).

4.10 Effect of DMSO on the proliferative response stimulated by PMA in synergy with anti-CD3.

Figure 4.10 shows that DMSO at concentrations from 50 to 200mM dose-dependently inhibited proliferation of PBMC stimulated with anti-CD3, 0.1μg/ml, plus PMA, 0.3nM. This effect ranged from about 13% below control at 50mM to about 60% below control at 200mM. Control samples contained cells stimulated with PMA + anti-CD3, without DMSO. The concentration of DMSO needed to inhibit proliferation by 50% was 162.53 ± 21.36mM.

4.11 Effect of DMSO on IL-2 release stimulated by PMA in synergy with anti-CD3.

Figure 4.11 shows that DMSO, 50-200mM, dose-dependently inhibited PMA+anti-CD3-induced IL-2 release. This effect ranged from about 20% below control at 50mM to about 60% below control at 200mM. The concentration of DMSO needed to promote 50% inhibition of the control response (197.65 ± 5.54mM) was similar to that needed to inhibit proliferation (Figure 4.10).

4.12 Effect of DMSO on free radical production stimulated by PMA in synergy with anti-CD3.

Figure 4.12 shows that DMSO, 100mM, inhibits PMA, 0.3nM, plus anti-CD3, 0.1μg/ml, -induced DCF fluorescence. 35 minutes after the addition of stimulus, the DCF fluorescence of DMSO-treated cells was 67% lower than untreated cells.

DMSO acts primarily on the hydroxyl radical. The concentrations of DMSO promoting about 50% inhibition of the control proliferation, IL-2 release and ROS generation, were similar in magnitude, suggesting a major role for this radical in PMA + anti-CD3-mediated signalling pathways.

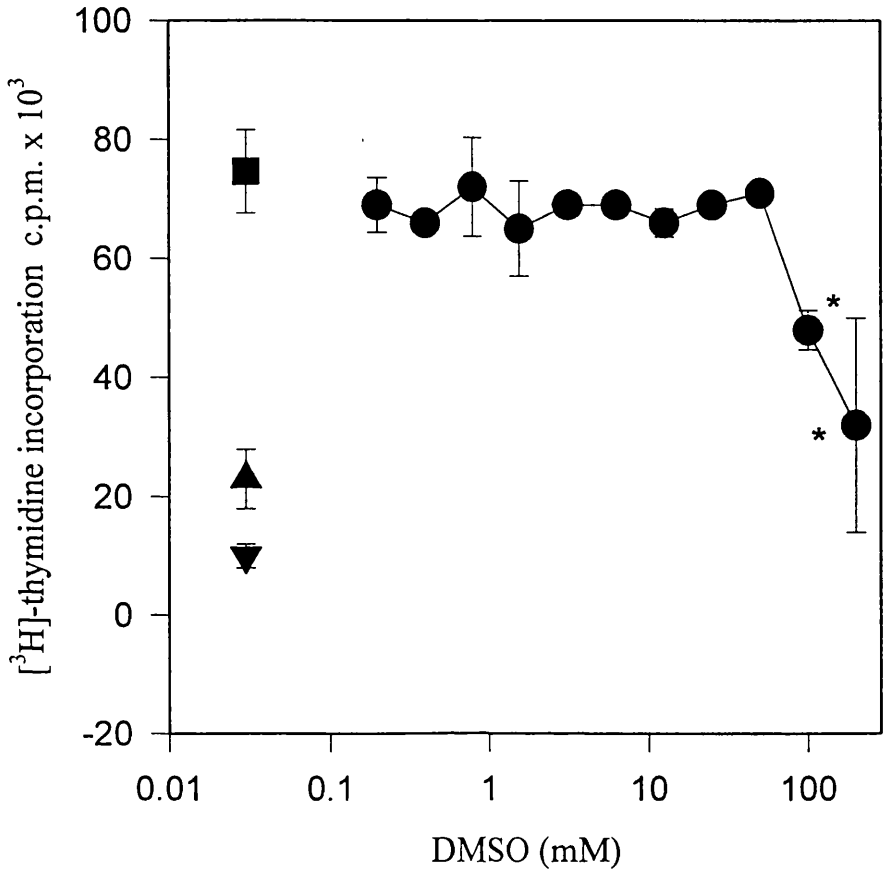


Figure 4.10
The effect of DMSO on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of DMSO on proliferation of PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were treated with: ▼ PMA, 0.3nM alone; ▲ anti-CD3, 0.1μg/ml alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + DMSO at the concentrations shown. [^{3}H]-thymidine was added for the last 16 hours of the 48 hour culture. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of DMSO, using the Student's t test. The IC_{50} of DMSO was 162.53 ± 21.36 mM.

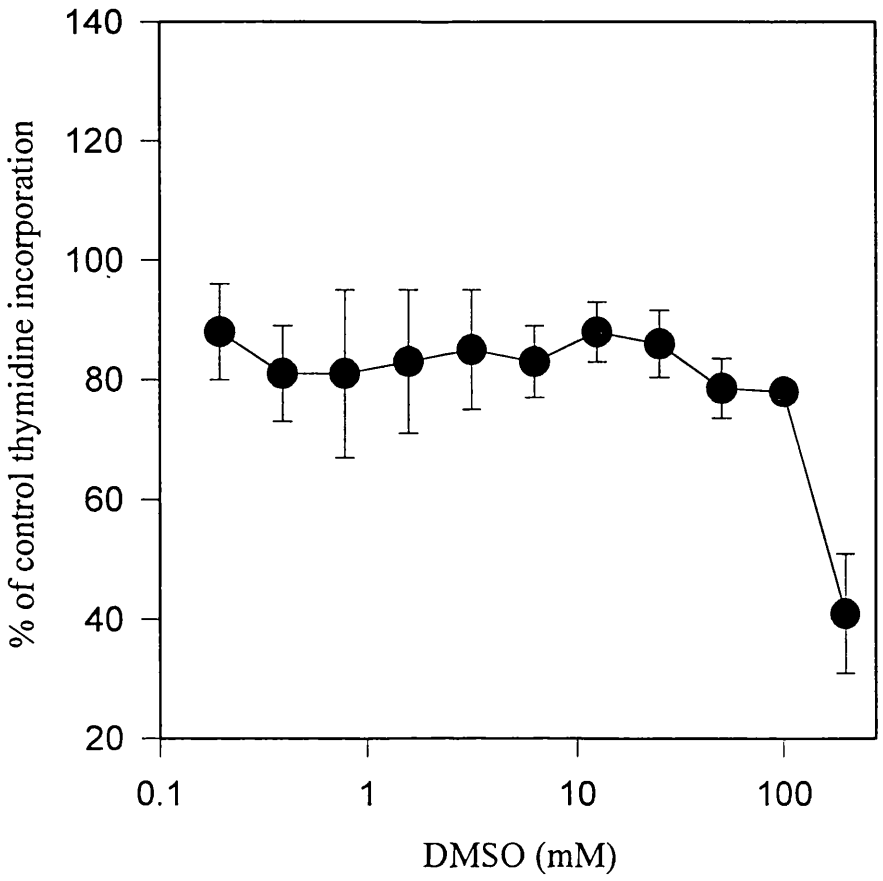


Figure 4.11
The effect of DMSO on IL-2 release by PBMC.
The concentration-response relationship for the effect of DMSO on IL-2 production by PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. PBMC were incubated with DMSO at the concentrations shown. PMA and anti-CD3 were added simultaneously. After 48 hours, supernatants were aspirated and IL-2 was determined in a proliferation assay using CTLL. Results are expressed as % of the control thymidine incorporation in the control sample (ie. PMA + anti-CD3 in the absence of DMSO), equivalent to 2.5ng/ml IL-2. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. The IC_{50} of DMSO was 197.65 ± 5.54 mM.

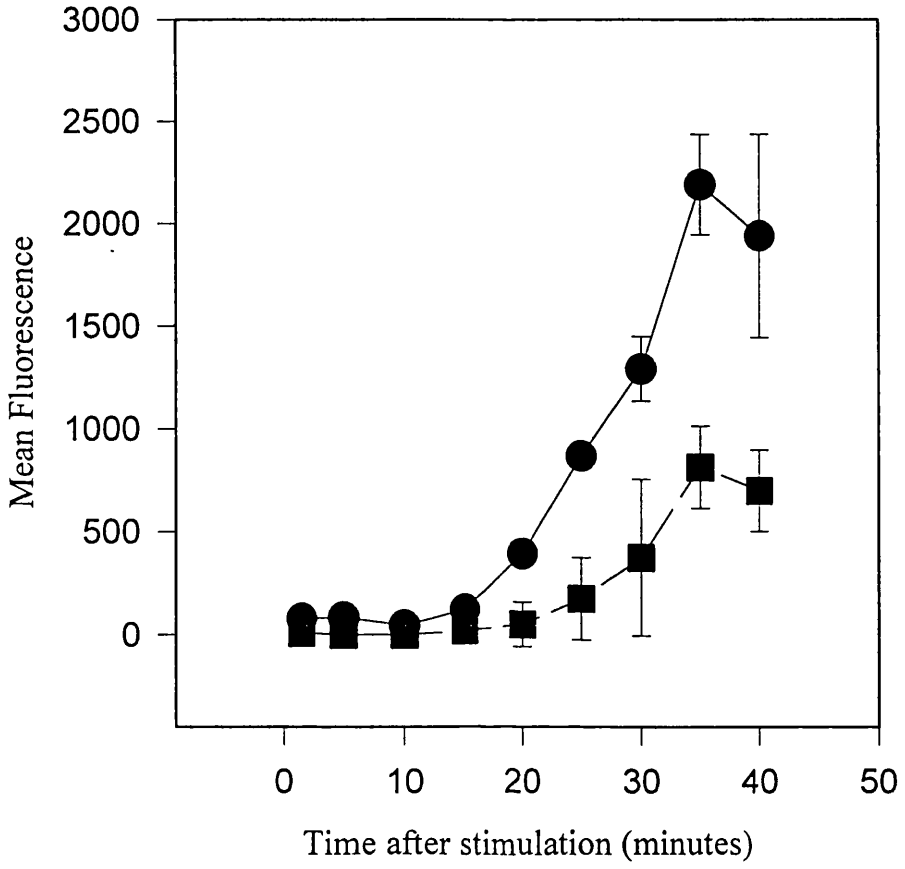


Figure 4.12
The effect of DMSO on the DCF fluorescence of PBL.
The effect of DMSO on ROS production by the lymphocyte population from PBMC, stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were treated with: ● medium alone; ■ 100mM DMSO; 30 minutes before the addition of stimulus. Fluorescence data was collected every 5 minutes for a 40 minute period. The data are the mean ± s.e.m. from three separate experiments. 2-way ANOVAs were used to statistically analyse the data. The p value obtained for no DMSO vs 100mM DMSO, following a 20 minute delay, was 0.019.

4.13 Effect of vitamin E on the proliferative response stimulated by PMA in synergy with anti-CD3.

Figure 4.13 shows that vitamin E, 0.19-3mM, produced a concentration-dependent inhibition of cell proliferation. At concentrations of vitamin E less than 0.19mM the effects of vitamin E on thymidine incorporation were statistically insignificant ($p>0.05$). The inhibitory effect ranged from about 6% below control at 0.19mM to virtually complete abrogation of the proliferative response at 3mM. The concentration of vitamin E needed to promote 50% inhibition of the control thymidine incorporation was 0.60 ± 0.05mM.

4.14 Effect of vitamin E on the IL-2 release stimulated by PMA in synergy with anti-CD3.

Figure 4.14 shows that vitamin E, 0.01-3mM, significantly inhibited PMA + anti-CD3-induced IL-2 release. This effect ranged from about 10% below control at 0.01mM to about 95% below control at 3mM. The concentration of vitamin E needed to inhibit IL-2 release and proliferation by 50 % of its control value were similar; 0.79 and 0.6mM respectively.

4.15 Effect of vitamin E on free radical production stimulated by PMA in synergy with anti-CD3.

Unlike the action of vitamin E on proliferation and IL-2 release, vitamin E had no significant effect on free radical production stimulated by PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Figure 4.15 shows that vitamin E, 0.8mM, produced no significant change in the DCF fluorescence over the entire 40 minute period. Vitamin E, 0.8mM, inhibited proliferation by more than 50% below control value.

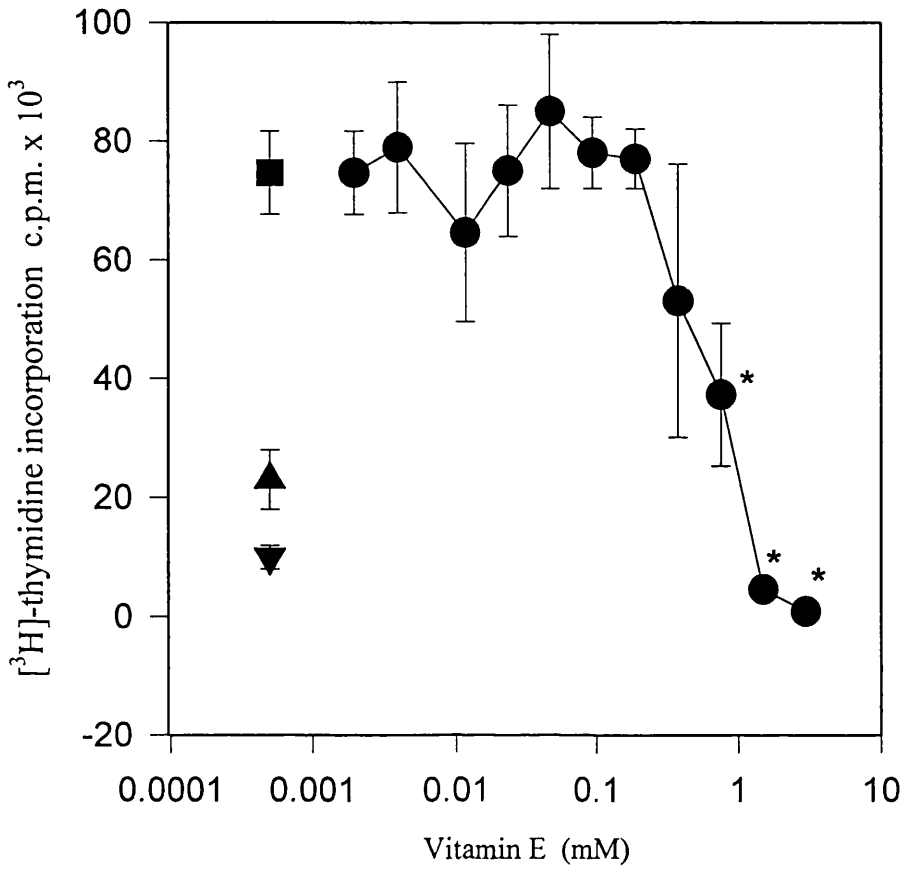


Figure 4.13
The effect of vitamin E on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of vitamin E on proliferation of PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. PBMC were incubated with vitamin E at the concentrations shown. PMA and anti-CD3 were added simultaneously and the cells were cultured for 48 hours prior to harvesting. ▼ PMA, 0.3nM, alone; ▲ anti-CD3, 0.1μg/ml alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + vitamin E. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of vitamin E. The IC_{50} of vitamin E was 0.60 ± 0.05 mM.

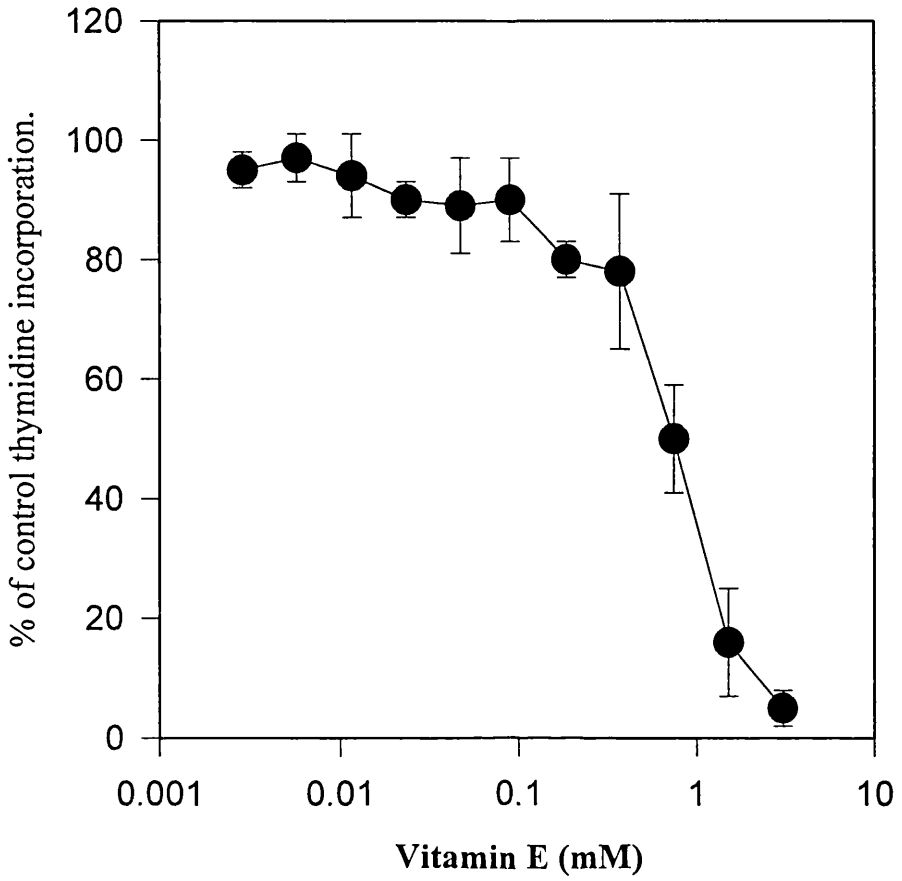


Figure 4.14
The effect of vitamin E on IL-2 release by PBMC.
The concentration-response relationship for the effect of vitamin E on IL-2 production by PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. PBMC were incubated with vitamin E at the concentrations shown. PMA and anti-CD3 were added simultaneously and the cells cultured for 48 hours prior to the collection of supernatants for IL-2 assay. Results are expressed as the % of the thymidine incorporation in the control sample (PMA + anti-CD3 in the absence of vitamin E), equivalent to 2.5ng/ml IL-2. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. The IC_{50} of vitamin E was 0.79 ± 0.04 mM.

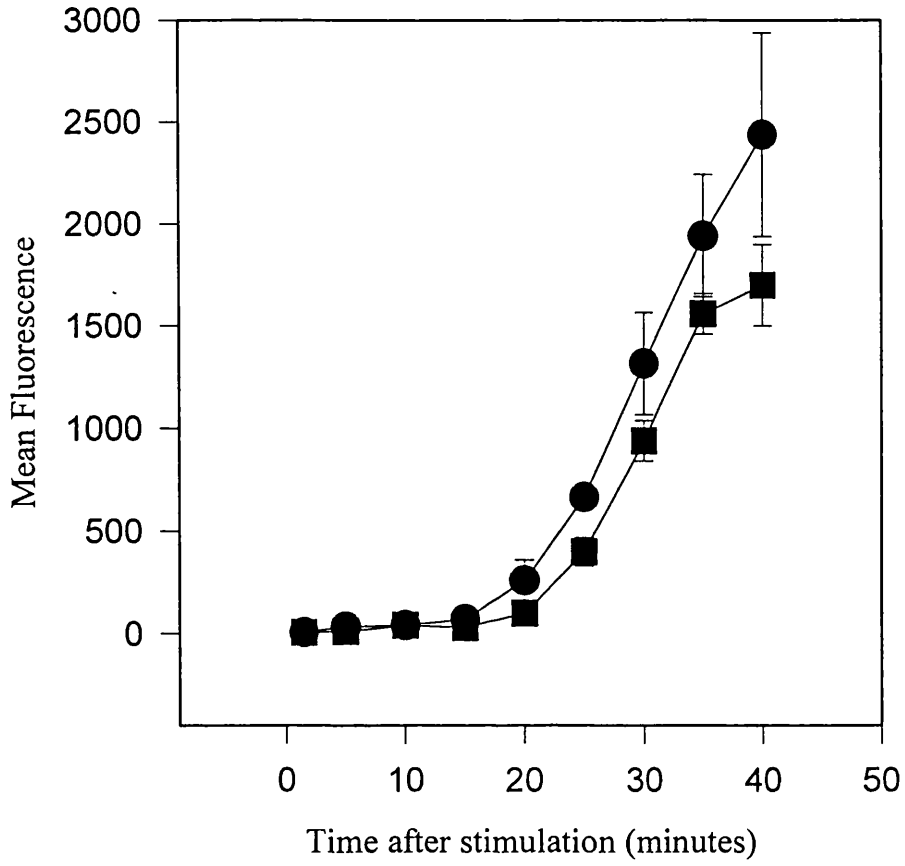


Figure 4.15
The effect of vitamin E on DCF fluorescence of PBL.
The effect of vitamin E on ROS production by the lymphocyte population from PBMC, stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. 30 minutes prior to the addition of stimulus cells were treated with: ● medium alone; ■ 0.8mM vitamin E. Mean fluorescence readings were taken every 5 minutes and subtracted from parallel cultures in the absence of PMA and anti-CD3. The results show the mean ± s.e.m from three separate experiments.

4.16 Effect of NAC on the proliferative response stimulated by PMA in synergy with anti-CD3.

Figure 4.16 shows that NAC, 0.05-50mM, dose-dependently inhibited proliferation. This effect was significantly different ($p<0.05$) from control thymidine incorporation at concentrations of NAC greater than or equal to 1mM. The concentration of NAC needed to promote 50% inhibition of the control thymidine incorporation was 2.50 ± 0.13mM.

Note: Effect of NAC on IL-2 release.

At the concentrations used, NAC affected CTLL growth in the presence of a fixed concentration of recombinant human IL-2 (see Figure 2.2). Hence the CTLL assay could not be used to determine the effect of NAC on IL-2 release by PBMC.

4.17 Effect of NAC on free radical production stimulated by PMA in synergy with anti-CD3.

As for vitamin E, NAC inhibited proliferation in a dose-dependent manner but failed to inhibit free radical production. Figure 4.17 shows NAC, 5mM, had no significant effect on the DCF fluorescence over the entire 40 minute period studied. NAC, 5mM, inhibited proliferation by about 85% below control.

A summary of these results is presented in Table 6.1 at the end of chapter 6.

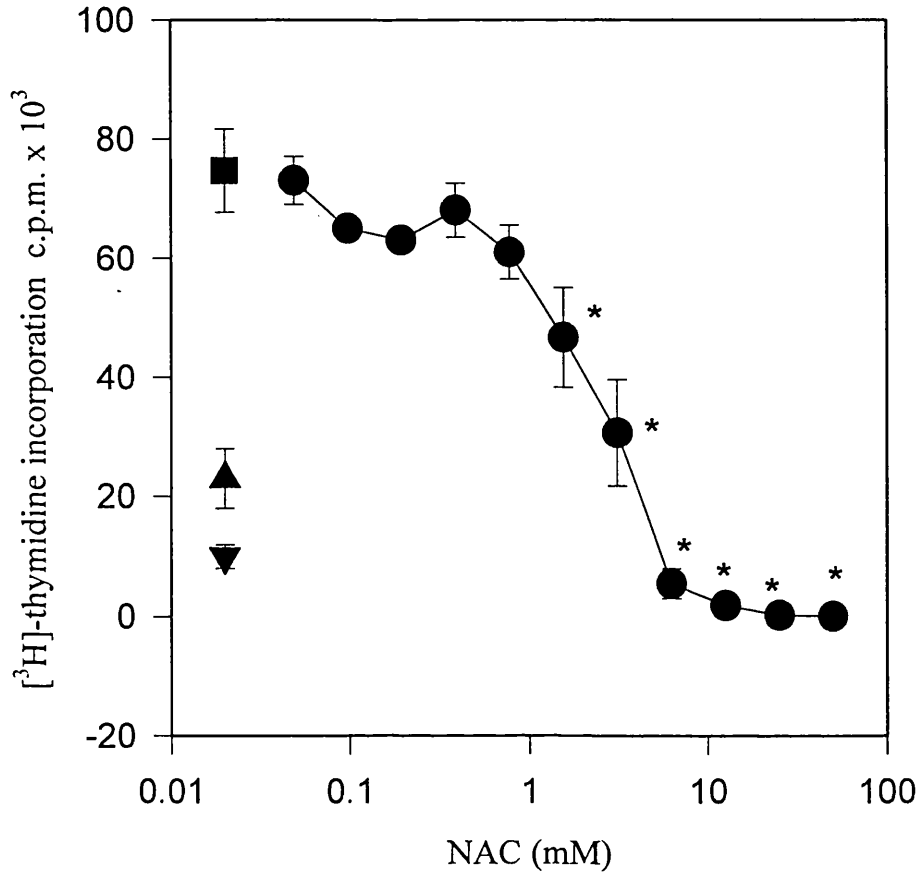


Figure 4.16
The effect of NAC on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of NAC on proliferation of PBMC stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. PBMC were incubated with NAC at the concentrations shown. PMA and anti-CD3 were added simultaneously and the cells were incubated for 48 hours prior to the measurement of thymidine incorporation. ▼ PMA, 0.3nM alone; ▲ anti-CD3, 0.1μg/ml alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + NAC. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<.0.05$) from control values in the absence of NAC. The IC_{50} of NAC was 2.50 ± 0.13 mM.

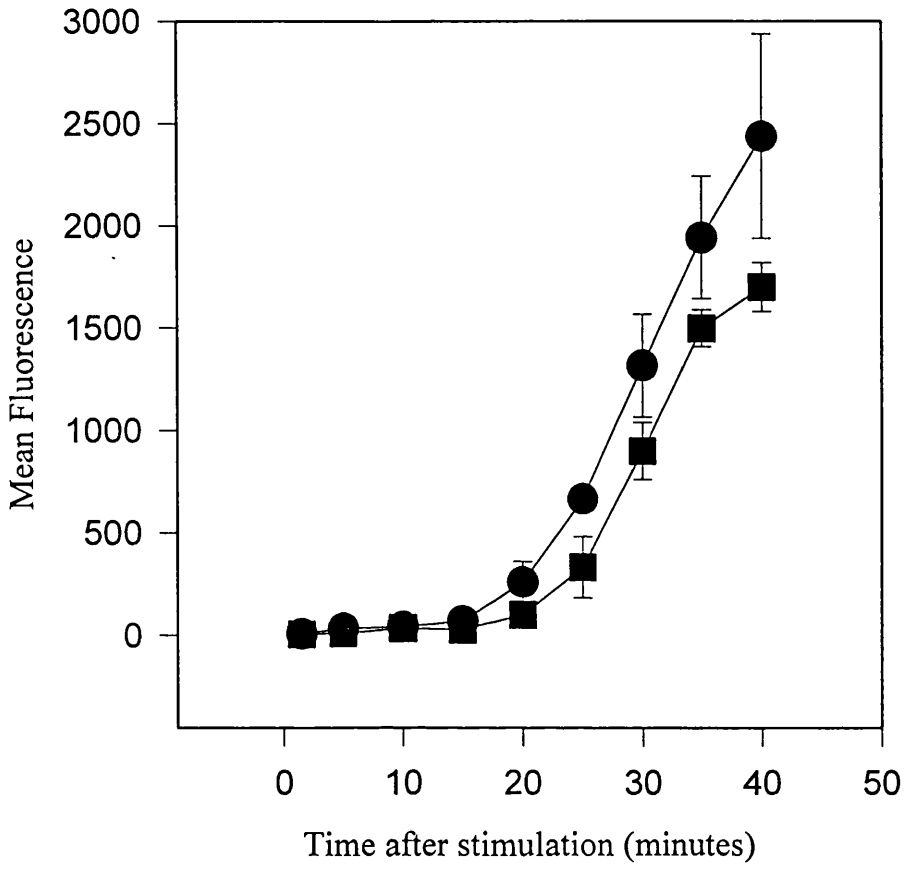


Figure 4.17
The effect of NAC on the DCF fluorescence of PBL.
The effect of NAC on ROS production by the lymphocyte population from PBMC, stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were pretreated with (●) medium or (■) NAC, 5mM, 30 minutes prior to the addition of stimulus. Mean fluorescence readings were taken every 5 minutes for 40 minutes and subtracted from parallel cultures in the absence of PMA and anti-CD3. The data are the mean ± s.e.m. from three separate experiments.

Discussion.

The primary aim of the experiments in this chapter was to replace the calcium ionophore (used in chapter 3) with a more physiological tool to increase the intracellular calcium concentration. This was done using the anti-CD3 mAb $UCHT_1$. The role of ROS in the activation pathways stimulated by a combination of PMA and anti-CD3 for T cell activation was then studied.

The T cell receptor for antigen comprises an α-β heterodimer associated non-covalently with at least five different subunits of the CD3 antigen (Weiss *et al.*, 1987). The functional role of CD3 is not clear, but previous studies have suggested that it may be involved in T cell activation. Interaction of CD3 with its receptor results in an increased intracellular calcium concentration (O'Flynn *et al.*, 1985), thus providing one of the two activation signals needed for a full activation response. In the experiments performed in this chapter, the second signal was provided by PMA, by its action on protein kinase C.

In agreement with previous studies (Weiss *et al.*, 1984), the results showed that T cells in a mixed population from peripheral blood leukocytes can be activated by anti-CD3, 0.1μg/ml, alone, as measured by proliferation and IL-2 assays, but that a much larger response is observed when used in combination with PMA, 0.3nM, (Figure 4.1, 4.2). How the increase in calcium and protein kinase C activation result in IL-2 gene regulation and T cell proliferation is unclear and whether it occurs by a mechanism similar to that seen with PMA and A23187 remains to be established.

Since PMA and anti-CD3 synergised for IL-2 release and proliferation (as seen with PMA plus A23187) it was anticipated that this series of experiments would also be paralleled by an increase in free radical production.

Anti-CD3, 0.1μg/ml, alone had little effect on ROS formation. PMA, 0.3nM, alone produced a reproducible ROS signal which was about 25 times greater than for anti-CD3 alone. However, a combination of the two agents enhanced DCF fluorescence, generating a much larger ROS signal than the summed response to either stimulus alone. This data in association with that of chapter 3, suggest that T cell proliferation

stimulated by PMA plus A23187 or anti-CD3 is accompanied by free radical production.

To further investigate the relationship between ROS generation and T cell activation, a series of compounds whose common feature was their ability to prevent formation of, or inactivate ROS were used. In addition to the antioxidants, desferrioxamine and ascorbic acid, used in chapter 3, three other compounds were used; dimethylsulfoxide (DMSO), N-acetyl cysteine (NAC) and vitamin E (α-tocopherol).

It is evident from Figures 4.4, 4.5, 4.10, 4.13 and 4.16, that there is a dose-dependent inhibition of the proliferative response by the antioxidants, as assessed by the incorporation of thymidine into DNA 48 hours post-stimulation with PMA plus anti-CD3. These results are in agreement with other studies (Chaudri *et al.*, 1986; 1988; Hunt *et al.*, 1989). All antioxidants were without effect on trypan blue exclusion at the highest concentration tested, thus confirming that the inhibition was not related to a toxic action of these compounds.

In contrast to the effects of the antioxidants on IL-2 release stimulated by PMA plus A23187 (chapter 3), the antioxidants were effective at inhibiting PMA plus anti-CD3-induced cytokine expression. The fact that the antioxidants did not affect the growth of cells already expressing IL-2 receptors (CTLL) confirms that they did not interfere with the assay of IL-2 and suggests they may act at a step up-stream of the expression of the IL-2 receptor. These data imply that the ROS-dependent event in T cell activation occurs within a few hours of activation. The nature of the "site" of action of these radical species, however, cannot be deduced from these results.

There are a number of ways in which oxidative events could influence cellular activity. Glutathione status appears to be critical for proliferation (Fidelus, 1988) and intracellular glutathione is an important determinant of cellular redox status (Hunt and Stocker, 1990). Intracellular redox events influence the activity of some enzymes, through alterations in the oxidation state of cysteine residues by a complex mechanism involving thiol/disulphide exchange (Staal *et al.*, 1993). ROS may influence intracellular glutathione (GSH) through glutathione peroxidase, generating GSSG, and ultimately altering the GSH:GSSG equilibrium. It is possible, therefore,

that ROS may influence cellular events indirectly through its influence on GSH levels.

Interaction of IL-2 with the IL-2 receptor is vital for T cells to undergo cell division (Smith, 1988). In accordance with the suggestion that inhibition of IL-2 release ultimately inhibits proliferation, it was found that concentrations of the antioxidants needed to inhibit IL-2 release by 50% of control were similar to concentrations needed to promote 50% inhibition of proliferation. However, the IC_{50} values for proliferation and IL-2 release were several orders of magnitude higher than those required for inhibition of ROS production, as shown in Table 6.1 (chapter 6). This suggests that although ROS formation can be identified in T lymphocytes, it is not likely to be the sole pathway for the progression of lymphocytes onto the DNA synthetic phase of the cell cycle. Another possibility which cannot be excluded, is that these compounds may be exerting effects on cellular signalling and gene expression that are unrelated to their antioxidant properties. This suggestion was made earlier with reference to desferrioxamine in chapter 3.

Of particular interest in this chapter are the data obtained using vitamin E and NAC. Vitamin E and NAC dose-dependently inhibited proliferation of PBMC but had no effect on the ROS production, as assessed by a DCF fluorescence change. The role of vitamin E as an antioxidant agent preventing peroxidation of unsaturated fatty acids by scavenging lipophilic radicals within membranes is widely recognised (Packer and Landvik, 1989). Vitamin E dose-dependently inhibited proliferation and IL-2 release by PBMC at concentrations of similar magnitude, but was unable to block free radical production. This could be explained in several ways. Firstly, vitamin E is a lipophilic compound which slowly diffuses into the cell. During a 48 hour culture period it remains in contact with the cell for longer periods of time than the 15 minute incubation used in the DCF assay, hence allowing greater time for it to diffuse into the cell. A second possibility is that vitamin E is acting by a reaction mechanism additional to its antioxidant properties. Boscoboinik *et al.,* (1991) demonstrated that vitamin E inhibits cell proliferation by preventing protein kinase C translocation to

the membrane and this enzyme has been shown to be important for T cell activation (Isakov and Altman, 1987).

NAC dose-dependently inhibited proliferation by PBMC but failed to block the ROS signal. The lack of effect on the ROS signal is consistent with the data produced using vitamin E. As with vitamin E this may be due to NAC accessibility to its target site or an additional action unrelated to its antioxidant properties. Despite its well-known antioxidant properties in vitro, the oxidant scavenging capacity of NAC is less well studied. NAC has been reported to act as a precursor of glutathione by increasing the cellular levels of cysteine, which may be rate-limiting in the synthesis of glutathione (Meister, 1983). The cysteine residue can be used for glutathione synthesis after the deacetylation of NAC. Glutathione is a major intracellular redox buffer of oxidative stress (Arnoma *et al.,* 1989). The results presented here do not allow a deduction of whether NAC exerted its effects through glutathione or oxidant scavenging.

NAC, 0.048- 50mM, enhanced CTLL growth in the presence of a fixed concentration of human r-IL-2. Therefore, the effects of NAC on IL-2 release by PBMC, stimulated with PMA and anti-CD3 could not be determined using the CTLL assay. Thus no data was obtained on the effects of NAC on IL-2 release.

Whilst concentrations of desferrioxamine, ascorbic acid, NAC and vitamin E were without effect on ROS formation, or several orders of magnitude lower than that needed to inhibit proliferation and IL-2 release by 50% of control, this was not seen with the data for DMSO. DMSO is known to be a scavenger of hydroxyl radicals (Novogrodsky *et al.,* 1982; 1986). The results presented in Figures 4.10, 4.11 and 4.12 show that the concentrations of DMSO needed to inhibit IL-2 release (IC_{50}=197mM), proliferation (IC_{50}=162mM) and ROS production (100mM inhibited by 67%) were similar in magnitude. In addition, the ROS signal was sensitive to the effects of desferrioxamine (Figure 4.8), which most probably acts on the hydroxyl radical through its chelation of iron; 0.01μM desferrioxamine reduced the DCF fluorescence to 73% below control. These data suggest a major role for the hydroxyl radical in the ROS signal generated by PMA plus anti-CD3. However, the results do not allow any deduction of the role that this radical species may play in the pathways

activated by PMA in synergy with anti-CD3 for T cell proliferation and IL-2 release. The fact that it is possible to block free radical formation and still allow T cell proliferation and IL-2 release, suggests the existence of a more "general target" for ROS as opposed to specific oxidative-sensitive "receptors".

From the results presented in this chapter it may be concluded that the synergistic effect between anti-CD3 and PMA for proliferation and IL-2 release is accompanied by an increase in free radical production.

The data show that the antioxidants inhibit lymphocyte activity, but that the mechanisms by which these compounds could be mediating inhibition of T cell proliferation, IL-2 release and ROS formation, could be at least partly different.

CHAPTER 5.

ROLE OF REACTIVE OXYGEN SPECIES IN THE T CELL ACTIVATION PATHWAYS STIMULATED BY ANTI-CD28 PLUS PMA OR ANTI-CD3

Introduction.

The experiments presented in this chapter were designed to investigate the T cell activation pathway stimulated by a CD28 monoclonal antibody (mAb). Although there is considerable evidence to suggest that CD28 acts as a co-receptor of T cell signalling (June *et al.*, 1994), the nature of the biochemical signals delivered by CD28 is still controversial. The rationale here was to study the role of ROS as potential "second messenger-like" molecules following anti-CD28 binding.

The first series of experiments were performed using a phorbol ester, PMA, and anti-CD28 mAb 913.12. Both stimuli were tested alone and in combination for proliferation, IL-2 release and ROS generation.

It has recently been suggested by Nunes *et al.*, (1995) that different CD28 mAbs have different actions on T cell functions. How these different antibodies mediate these different effects is not clear. It was interesting to see if different CD28 mAbs also differed in their ability to generate a ROS signal. The second anti-CD28 mAb (15E9) used was a gift from Dr. P. Beverly, ICRF, UCL.

It is well documented that while engagement of the T cell receptor triggers the initial steps of cell activation, it is not sufficient for a full activation response. Hence, anti-CD28 mAb 15E9 was used in combination with anti-CD3 mAb $UCHT_1$ as a model for T cell activation. These two stimuli were tested alone and in combination for proliferation and ROS production.

Additional experiments with the antioxidants, desferrioxamine and ascorbic acid, were performed to test if ROS had a role in CD28-mediated signalling.

5.1 Effect of anti-CD28 mAb 913.12 on PMA-induced proliferation.

The results in Figure 5.1 show that anti-CD28 mAb 913.12, 1/800 dilution, potentiated the response to a suboptimal concentration of PMA, 0.3nM, in inducing a proliferative response.

PMA alone and anti-CD28 mAb 913.12 alone had little effect on thymidine incorporation. A combination of PMA plus anti-CD28 enhanced the proliferative response by more than 80% relative to either stimulus alone.

5.2 Effect of anti-CD28 mAb 913.12 on PMA-induced IL-2 release.

Figure 5.2 shows that anti-CD28 mAb 913.12, 1/800 dilution, potentiated PMA-induced IL-2 release. Results show the thymidine incorporated by CTLL supported by a 1:8 dilution of the culture supernatant. As for proliferation, PMA and anti-CD28 alone had no significant effect on IL-2 release. A combination of the two increased IL-2 release by 85% relative to either stimulus alone.

5.3 Effect of anti-CD28 mAb 913.12 on PMA-induced free radical production.

Previously it was demonstrated that the synergistic effect between PMA plus A23187 (chapter 3) and PMA plus anti-CD3 (chapter 4) for proliferation and IL-2 release was also accompanied by an increased ROS production. However, as shown in Figure 5.3, anti-CD28 did not potentiate the PMA-induced ROS signal. Anti-CD28 mAb 913.12 alone produced a small increase in DCF fluorescence; maximum mean fluorescence change was 45 units. PMA alone produced a larger ROS signal than anti-CD28 alone; maximum mean fluorescence change was 450 units.

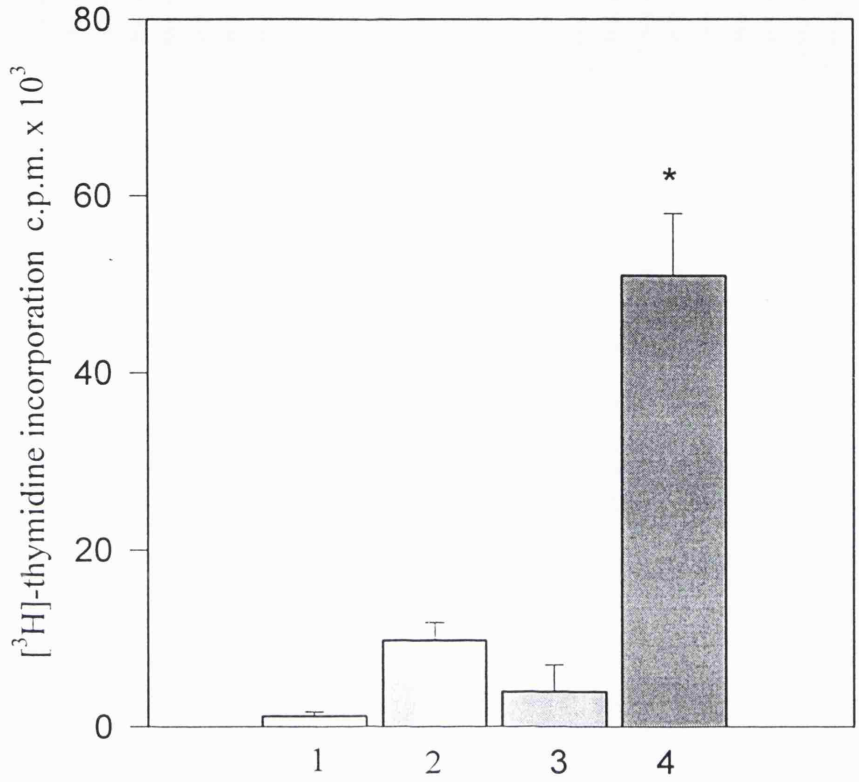


Figure 5.1
The influence of anti-CD28 on the PMA-induced thymidine incorporation.
The effect of anti-CD28mAb 913.12, 1/800 dilution, on proliferation of PBMC stimulated with PMA, 0.3nM. Cells were stimulated with: (1) medium alone; (2) PMA, 0.3nM alone ; (3) anti-CD28, 1/800 dilution alone; (4) PMA + anti-CD28. After 32 hours, a pulse of [^{3}H]-thymidine was added and cells were incubated for a further 16 hours. Each bar represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that the data is significantly different ($p<0.05$) to either stimulus alone.

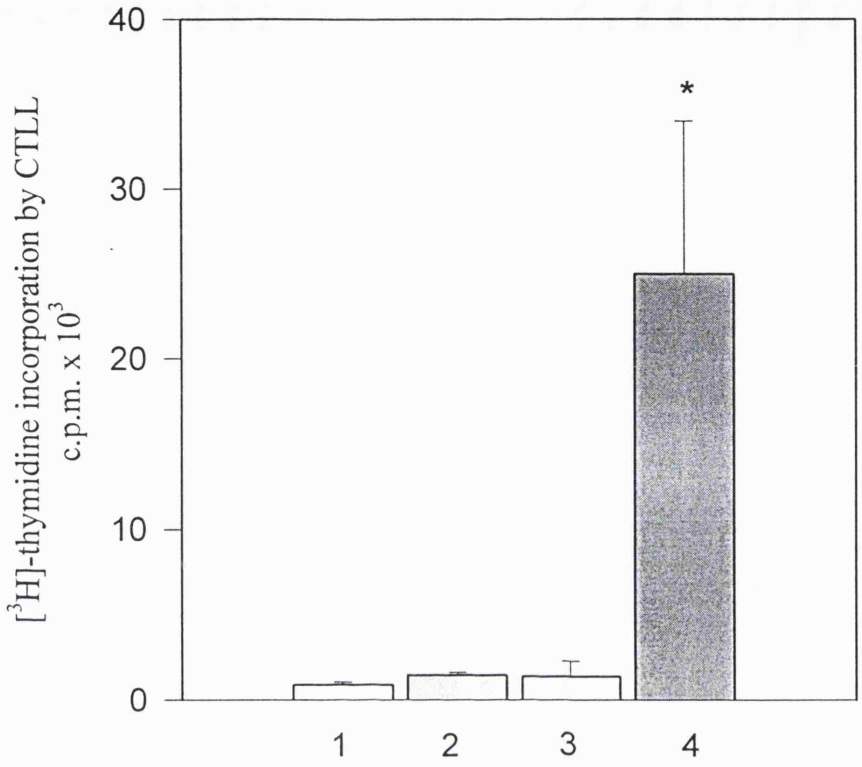


Figure 5.2
The influence of anti-CD28 on PMA-induced IL-2 release.
Effect of anti-CD28 mAb 913.12, 1/800 dilution, on IL-2 secretion by PBMC stimulated with PMA, 0.3nM. Cells were treated with: (1) medium alone; (2) anti-CD28, 1/800 dilution alone; (3) PMA, 0.3nM alone; (4) anti-CD28 + PMA. After 48 hours of culture, cell supernatants were collected and assayed for IL-2 using CTLL. Results show the thymidine incorporated by CTLL supported by a 1:8 dilution of the culture supernatant. Each bar represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that the data is significantly different ($p<0.05$) to either stimulus alone.

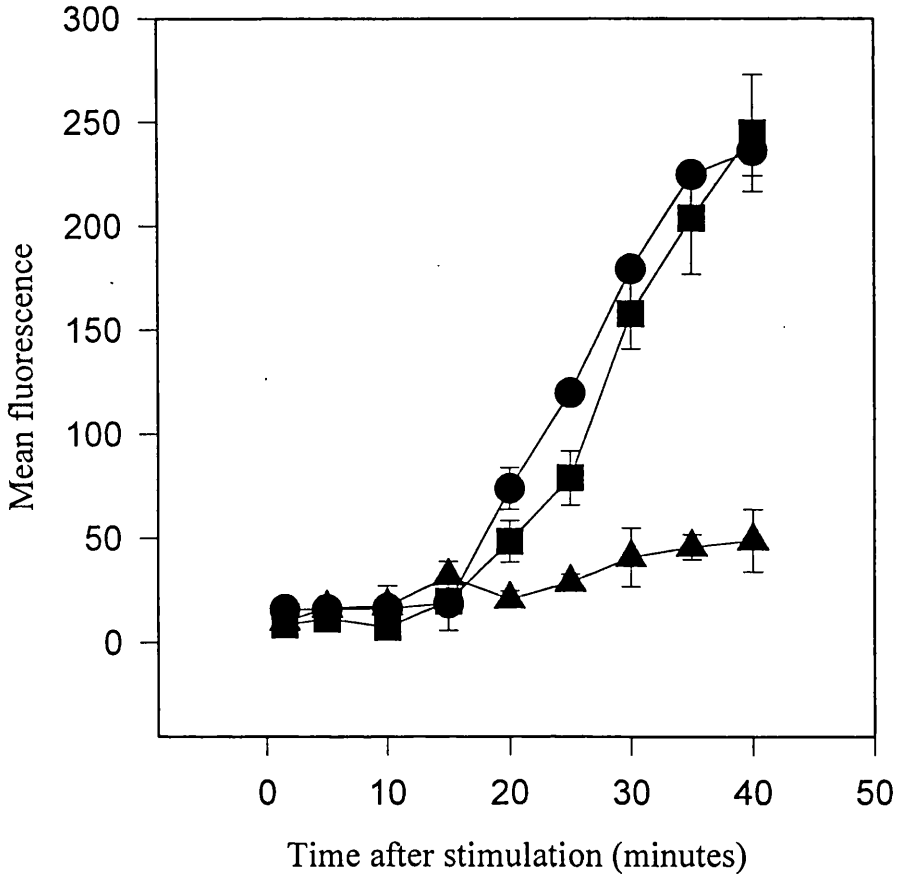


Figure 5.3
The influence of anti-CD28 on the DCF fluorescence from PMA-stimulated PBMC.
Effect of anti-CD28 mAb 913.12, 1/800 dilution, on ROS production by the lymphocyte population of PBMC, stimulated with PMA, 0.3nM. Cells were loaded with DCFH-DA, 10μM, 15 minutes before stimulation with: ● PMA, 0.3nM alone; ▲ anti-CD28 mAb 913.12, 1/800 dilution, alone; ■ PMA + anti-CD28. Fluorescence data was collected every 5 minutes and subtracted from parallel cultures in the absence of PMA and anti-CD28 at each time point. The data are the mean ± s.e.m. from three separate experiments.

Effect of antioxidants on the proliferative response stimulated by PMA in synergy with anti-CD28 mAb 913.12.

To investigate further whether ROS is involved in the synergistic action between PMA and anti-CD28mAb 913.12 for proliferation and IL-2 release, the sensitivity of these lymphocyte functions to the antioxidants, desferrioxamine and ascorbic acid, was studied. Neither antioxidant was cytotoxic, as determined by trypan blue exclusion, at the highest concentration of antioxidant used. Neither antioxidant interfered with the assay for IL-2, as assessed by their direct action on CTLL in the presence of a submaximal concentration of human r-IL-2.

5.4 Effect of desferrioxamine on proliferation.

Figure 5.4 shows that desferrioxamine, 0.024-50μM, inhibited proliferation in a dose-dependent manner. The concentration of desferrioxamine needed to promote 50% inhibition of the control thymidine incorporation was 0.99 ± 0.09μM.

From simple analysis of the inhibitory dose-response curve, the action of desferrioxamine appears to occur in a "two-step" manner. At concentrations of desferrioxamine less than 0.4μM, the effects of desferrioxamine on thymidine incorporation are statistically insignificant ($p>0.05$). However, concentrations of desferrioxamine greater than 0.4μM produced a sudden decline in the proliferative response. This effect was not seen using other models of T cell activation (PMA + A23187, or PMA + anti-CD3). In other models of T cell activation, increasing concentrations of desferrioxamine produced a steady decline in the proliferative response.

5.5 Effect of ascorbic acid on proliferation.

Figure 5.5 shows that ascorbic acid, 0.002-5mM, inhibited thymidine incorporation dose-dependently. This effect ranged from about 5% below control at 0.002mM to almost complete inhibition of proliferation at 5mM. The concentration of ascorbic acid that inhibited the control by 50% was 1.59 ± 0.12mM.

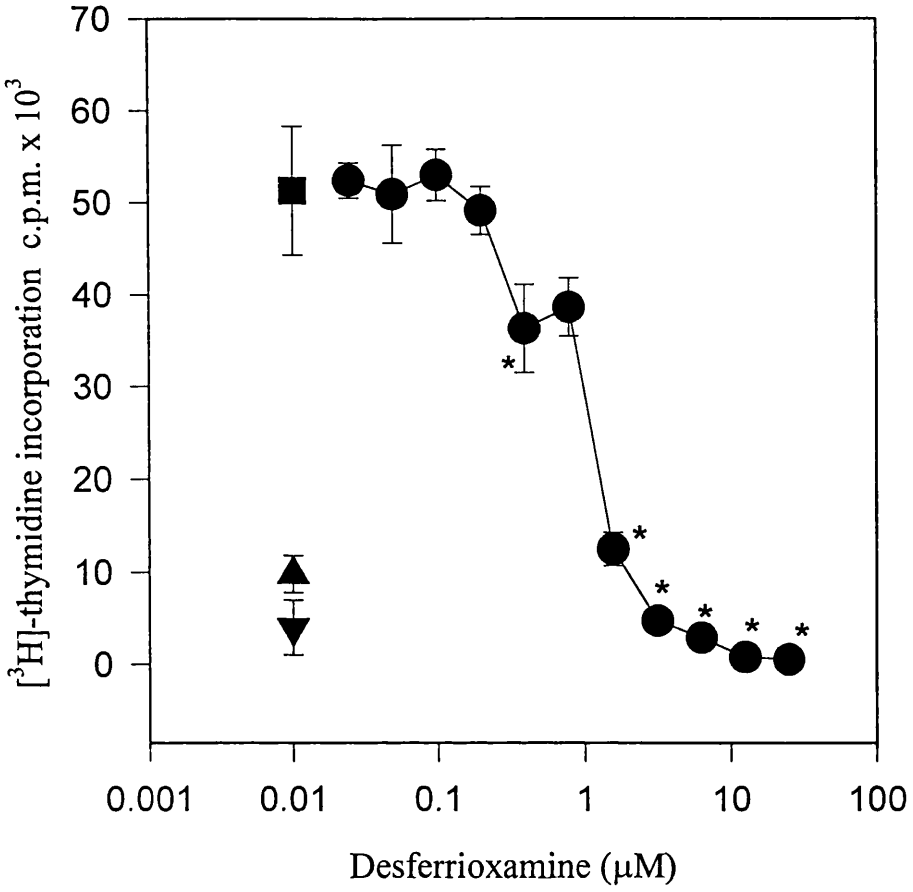


Figure 5.4
The effect of desferrioxamine on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of desferrioxamine on proliferation of PBMC stimulated with PMA, 0.3nM, plus anti-CD28 mAb 913.12, 1/800 dilution. Cells were incubated with desferrioxamine at the concentrations shown. PMA and anti-CD28 were added simultaneously. After 32 hours, cells were pulsed with [^{3}H]-thymidine and incubated for a further 16 hours. ▲ PMA, 0.3nM, alone; ▼ anti-CD28, 1/800, dilution alone; ■ PMA + anti-CD28; ● PMA + anti-CD28 + desferrioxamine. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of desferrioxamine, using the Student's t test. The IC_{50} of desferrioxamine was 0.99 ± 0.09 μM.

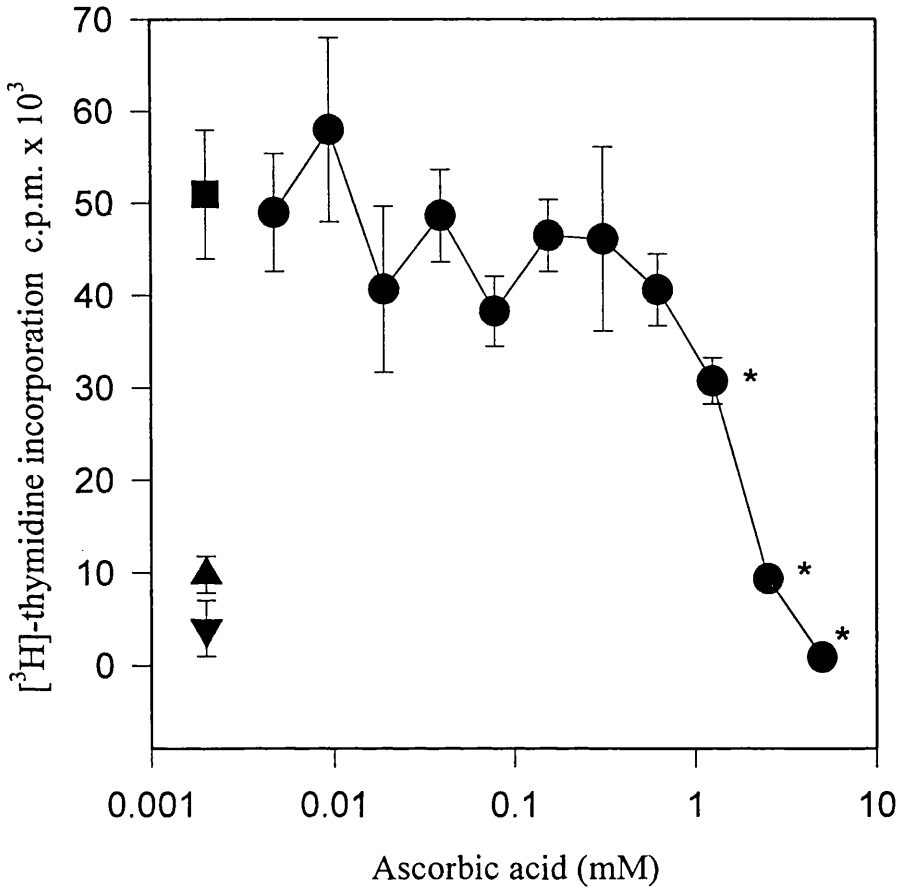


Figure 5.5
The effect of ascorbic acid on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of ascorbic acid on proliferation stimulated with PMA, 0.3nM, plus anti-CD28 mAb 913.12, 1/800 dilution. PBMC were incubated with ascorbic acid at the concentrations shown. PMA and anti-CD28 were added simultaneously. A pulse of [^{3}H]-thymidine was added for the last 16 hours of the 48 hour incubation period. ▲ PMA, 0.3nM alone; ▼ anti-CD28, 1/800 dilution alone; ■ PMA + anti-CD28; ● PMA + anti-CD28 + ascorbic acid. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of ascorbic acid, using the Student's t test. The IC_{50} of ascorbic acid was 1.59 ± 0.12 mM.

Effect of antioxidants on IL-2 release stimulated by PMA in synergy with anti-CD28 mAb 913.12.

Results are expressed as the percentage of the thymidine incorporated by CTLL supported by a 1:8 dilution of the control culture supernatant (cells stimulated by PMA + anti-CD28 without antioxidant).

5.6 Effect of desferrioxamine on IL-2 release.

Figure 5.6 shows that desferrioxamine from 0.024 to 50μM had little effect on IL-2 release stimulated by PMA, 0.3nM, plus anti-CD28 mAb, 1/800 dilution. Desferrioxamine at the highest concentration tested (50μM), inhibited the control IL-2 release by less than 50%.

5.7 Effect of ascorbic acid on IL-2 release.

Figure 5.7 shows that ascorbic acid, 0.002-5mM, inhibited IL-2 release dose-dependently. The concentration of ascorbic acid needed to promote 50% inhibition of the control IL-2 release was 0.61 ± 0.03mM.

The concentration of ascorbic acid needed to inhibit proliferation by 50% of the control was 62% higher than that needed to inhibit IL-2 release.

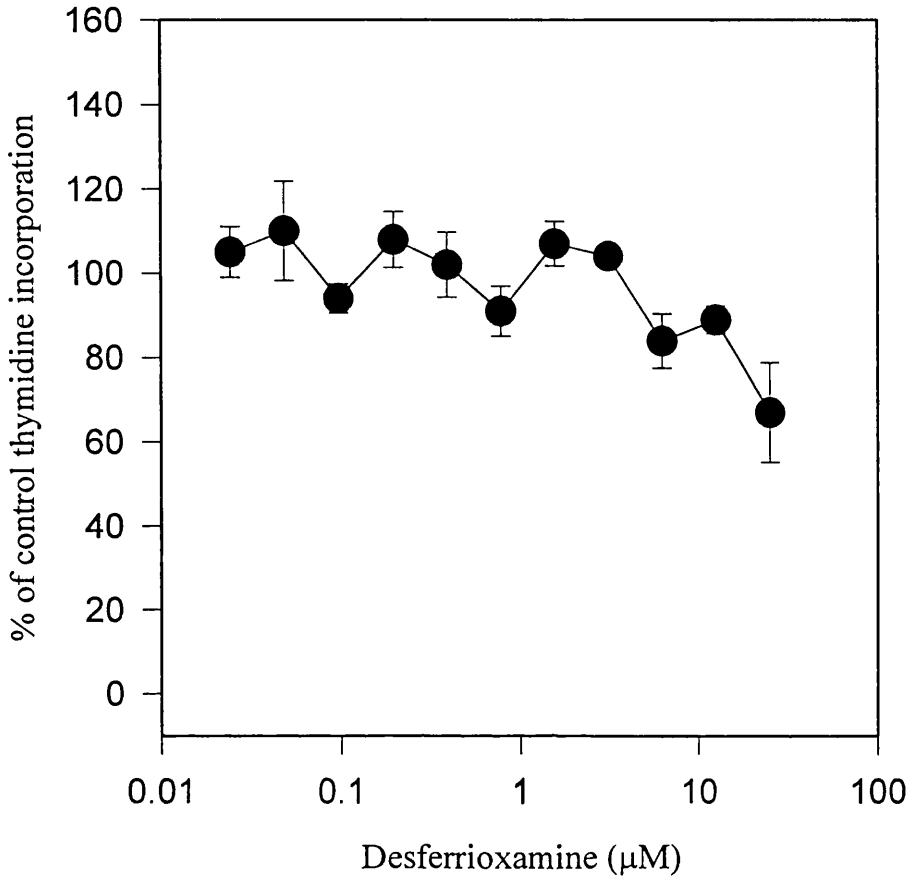


Figure 5.6
The effect of desferrioxamine on IL-2 release by PBMC.
The concentration-response relationship for the effect of desferrioxamine on IL-2 production by PBMC stimulated with PMA, 0.3nM, plus anti-CD28, 1/800 dilution. PBMC were incubated with desferrioxamine at the concentrations shown. PMA and anti-CD28 were added simultaneously and the cells were incubated for 48 hours prior to the collection of supernatants for IL-2 assay. Background levels of IL-2 release were less than 900 cpm, equivalent to less than 0.001ng/ml IL-2. Results are expressed as % of the control thymidine incorporation (ie. PMA + anti-CD28 in the absence of desferrioxamine) which was 31270 ± 4000 cpm and is equivalent to approximately 2.5ng/ml Il-2. PMA and anti-CD28 alone gave counts of less than 1500 cpm, equivalent to approximately 0.00063 ng/ml IL-2. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments.

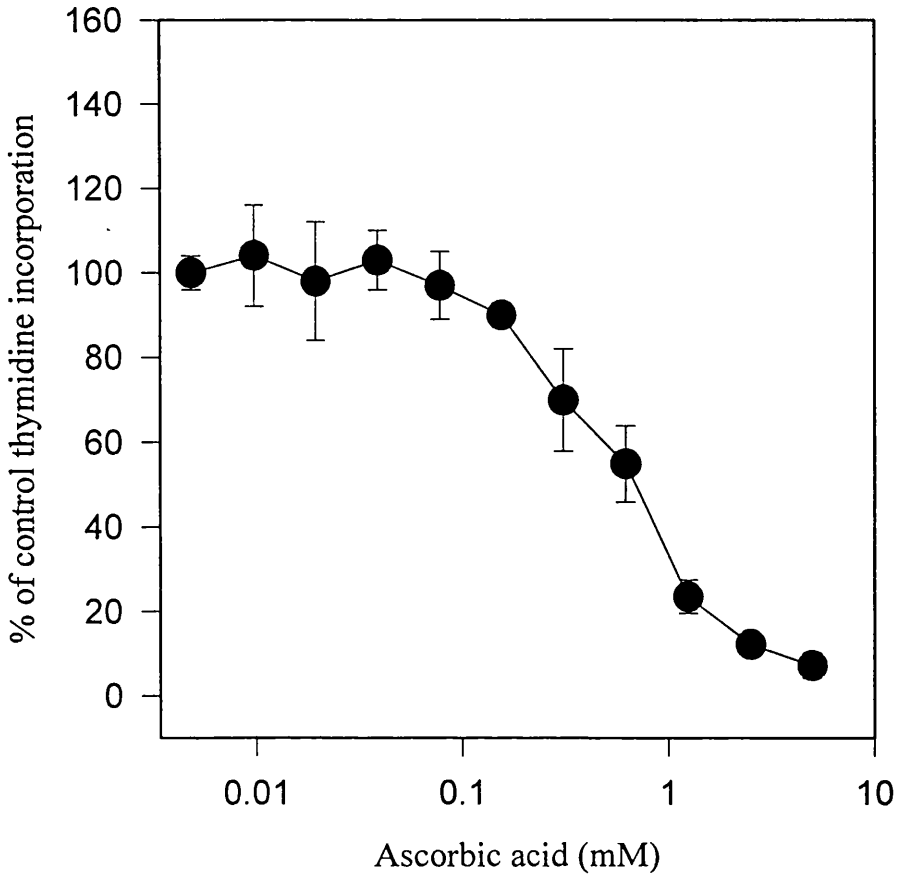


Figure 5.7
The effect of ascorbic acid on IL-2 release by PBMC.
The concentration-response relationship for the effect of ascorbic acid on IL-2 production by PBMC stimulated with PMA, 0.3nM, plus anti-CD28 mAb 913.12, 1/800 dilution. PBMC were incubated with ascorbic acid at the concentrations shown. PMA and anti-CD28 were added simultaneously. Cells were cultured for 48 hours prior to the collection of supernatants for IL-2 assay. Results are expressed as the % of the thymidine incorporation in control sample (PMA + anti-CD28 in the absence of ascorbic acid), equivalent to 2.5ng/ml IL-2. Each point represents the mean ± s.e.m. from three independent experiments. The IC_{50} of ascorbic acid was 0.61 ± 0.03 mM.

5.8 The effect of different anti-CD28 mAbs on free radical production.

To test whether different anti-CD28 mAbs elicit differing actions on T cell functions through changes in their ability to produce ROS, two different anti-CD28 mAbs were tested for free radical generation using flow cytometry. Anti-CD28 mAb 913.12, a rabbit anti-human antibody, was purchased from Serotec. It reacts with an antigen of 41KDa. Anti-CD28 mAb 15E9 was a gift from Dr. P. Beverly, ICRF, UCL. The exact nature of the epitope molecule identified by this antibody was unclear.

Figure 5.8 shows that the two monoclonal antibodies differ in their ability to produce a ROS signal. Anti-CD28 mAb 15E9 produced a much larger increase in DCF fluorescence compared to mAb 913.12; maximum mean fluorescence changes induced by mAb 15E9 and 913.12 was 223 and 49 units respectively. The ROS signal stimulated by anti-CD28 mAb 15E9 was linear over the first 30 minutes, reaching a maximum at 30 minutes, followed by a steady decline. The smaller ROS flux generated by anti-CD28 913.12 was not related to its concentration, since higher concentrations tested were unable to cause any further increase in DCF fluorescence. The difference in ROS generation by anti-CD28 mAb 15E9 and 913.12 was statistically significant ($p<0.05$) over the entire 40 minute period studied.

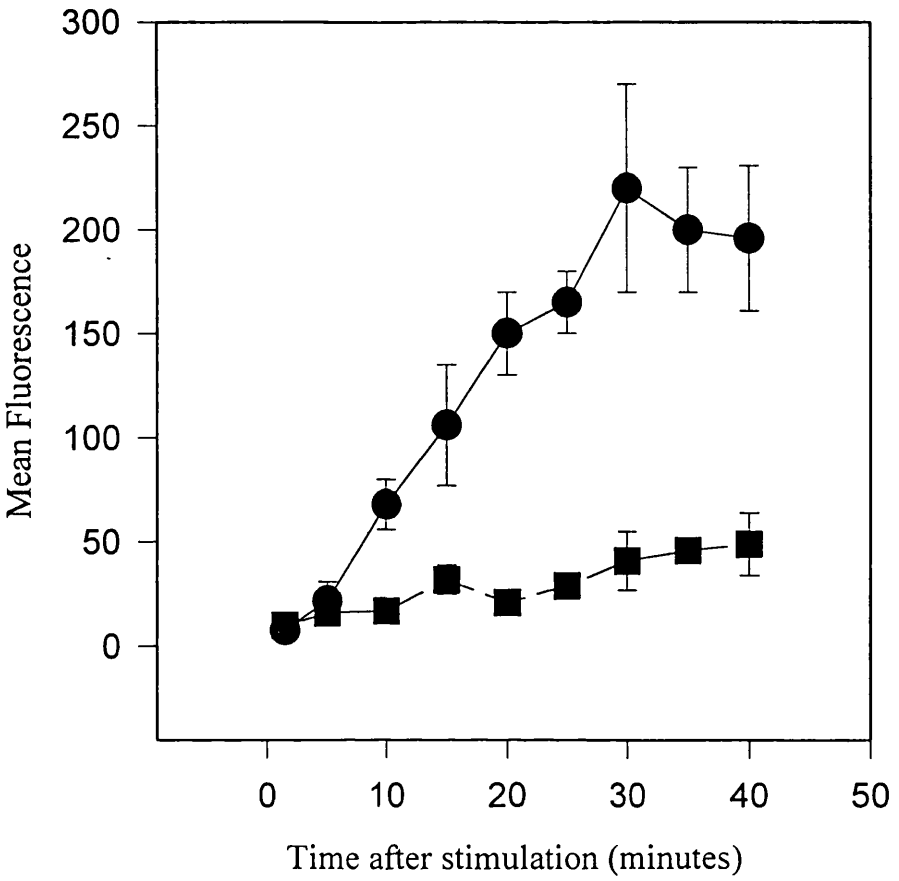


Figure 5.8
The effect of different anti-CD28 mAbs on the DCF fluorescence of PBL.
The effect of using two different anti-CD28 monoclonal antibodies on the ROS production by the lymphocyte population from PBMC. Cells were loaded with DCFH-DA, 10μM, 15 minutes prior to the addition of: ■ anti-CD28 monoclonal antibody 913.12, 1/800 dilution; ● anti-CD28 monoclonal antibody 15E9, 250ng/ml. Mean fluorescence readings were taken every 5 minutes and subtracted from parallel cultures in the absence of anti-CD28. The data are the mean ± s.e.m. from three separate experiments. 2-way ANOVAs were used to statistically analyse the data. The p value obtained for anti-CD28 monoclonal antibody, 913.12 vs 15E9 was less than 0.0001

Full T cell activation requires two signals (Lafferty *et al.*, 1985). Here, "two signals" were provided by anti-CD28 mAb 15E9 and a suboptimal concentration of anti-CD3 mAb $UCHT_1$.

5.9 Effect of anti-CD28 mAb 15E9 on anti-CD3-induced proliferation.

Figure 5.9 shows that anti-CD28 mAb 15E9,250ng/ml, potentiated the effect of anti-CD3, 0.1μg/ml, in inducing a proliferative response. Anti-CD3, 0.1μg/ml, alone induced a 78% larger thymidine incorporation than cells stimulated with anti-CD28 mAb 15E9, 250ng/ml, alone. A combination of the two antibodies increased proliferation by 60% relative to either stimulus alone.

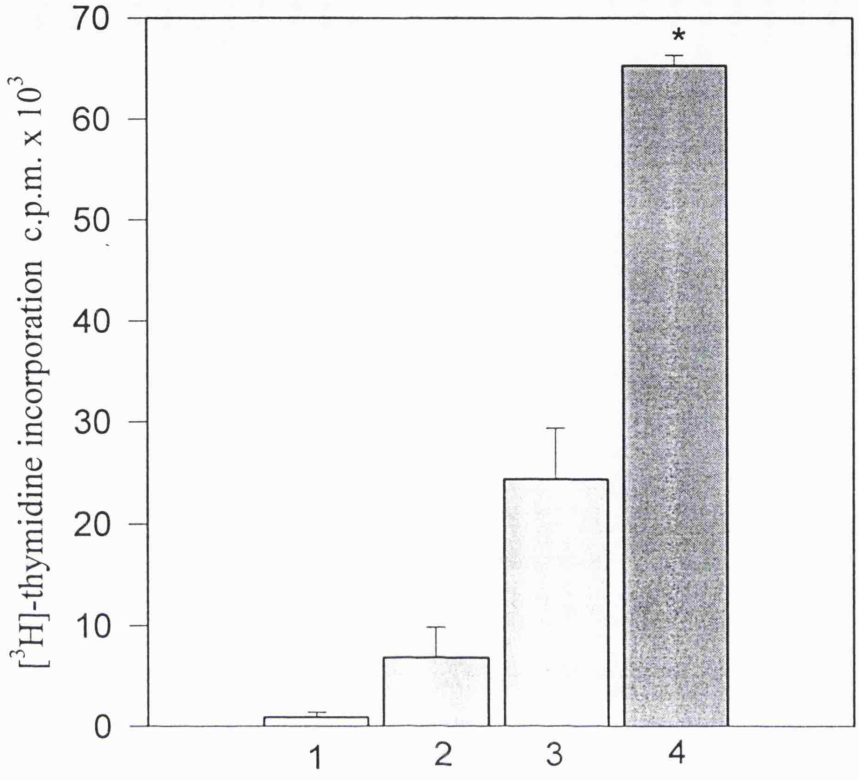


Figure 5.9
The influence of anti-CD28 mAb 15E9 on the anti-CD3-induced thymidine incorporation.
Effect of anti-CD28 mAb 15E9, 250ng/ml, on proliferation of PBMC stimulated with anti-CD3, 0.1μg/ml. Cells were treated with: (1) medium alone; (2) anti-CD28, 250ng/ml alone; (3) anti-CD3, 0.1μg/ml alone; (4) anti-CD28 + anti-CD3. Cells were pulsed with [^{3}H]-thymidine for the last 16 hours of the 48 hour period. Each bar represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that the data is significantly different ($p<0.05$) to either stimulus alone.

Effect of antioxidants on the proliferative response stimulated by anti-CD28 mAb 15E9 in synergy with anti-CD3.

In order to investigate the role of anti-CD28 mAb 15E9-induced ROS production on the potentiation of anti-CD3-induced proliferation, the sensitivity of the proliferative response to the antioxidants, desferrioxamine and ascorbic acid, was studied.

Control samples refer to the response from cells stimulated with anti-CD28 mAb 15E9 plus anti-CD3 without antioxidant.

5.10 Effect of desferrioxamine on proliferation.

Figure 5.10 shows that desferrioxamine, 0.024-50μM, inhibited proliferation dose-dependently. This effect ranged from about 8% below control at 0.02μM to about 94% below control at 50μM. The concentration of desferrioxamine needed to promote 50% inhibition of the control thymidine incorporation was 2.64 ± 0.27μM.

5.11 Effect of ascorbic acid on proliferation.

In contrast to the action of desferrioxamine, Figure 5.11 shows that ascorbic acid, 0.002-5mM, had little action on the proliferative response induced by anti-CD28 mAb 15E9 plus anti-CD3. This effect ranged from about 6% below control at 0.002mM to about 23% below control at 5mM.

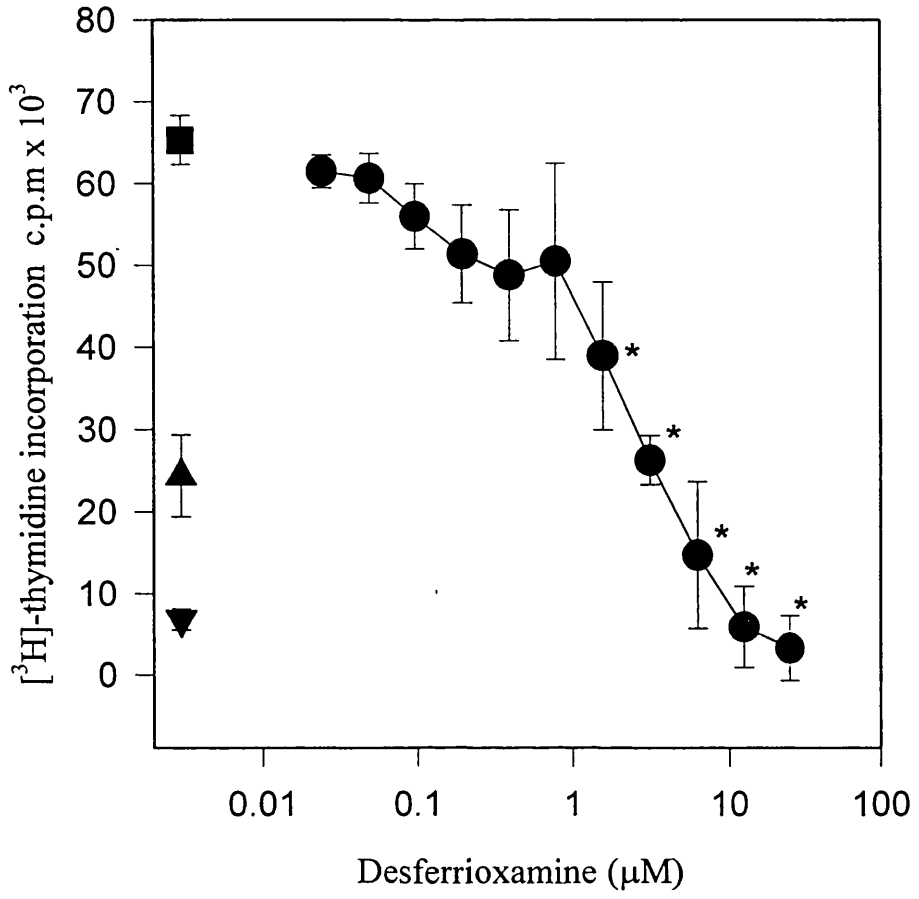


Figure 5.10
The effect of desferrioxamine on thymidine incorporation by PBMC.
The concentration-response for the effect of desferrioxamine on proliferation of PBMC stimulated with anti-CD28 mAb 15E9, 250ng/ml, plus anti-CD3, 0.1μg/ml. Cells were incubated with desferrioxamine at the concentrations shown. Anti-CD28 and anti-CD3 were added simultaneously and the cells were cultured for 48 hours prior to harvesting. ▼ anti-CD28, 250ng/ml, alone; ▲ anti-CD3, 0.1μg/ml, alone; ■ anti-CD28 + anti-CD3; ● anti-CD28 + anti-CD3 + desferrioxamine. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of desferrioxamine. The IC_{50} of desferrioxamine was 2.64 ± 0.27μM.

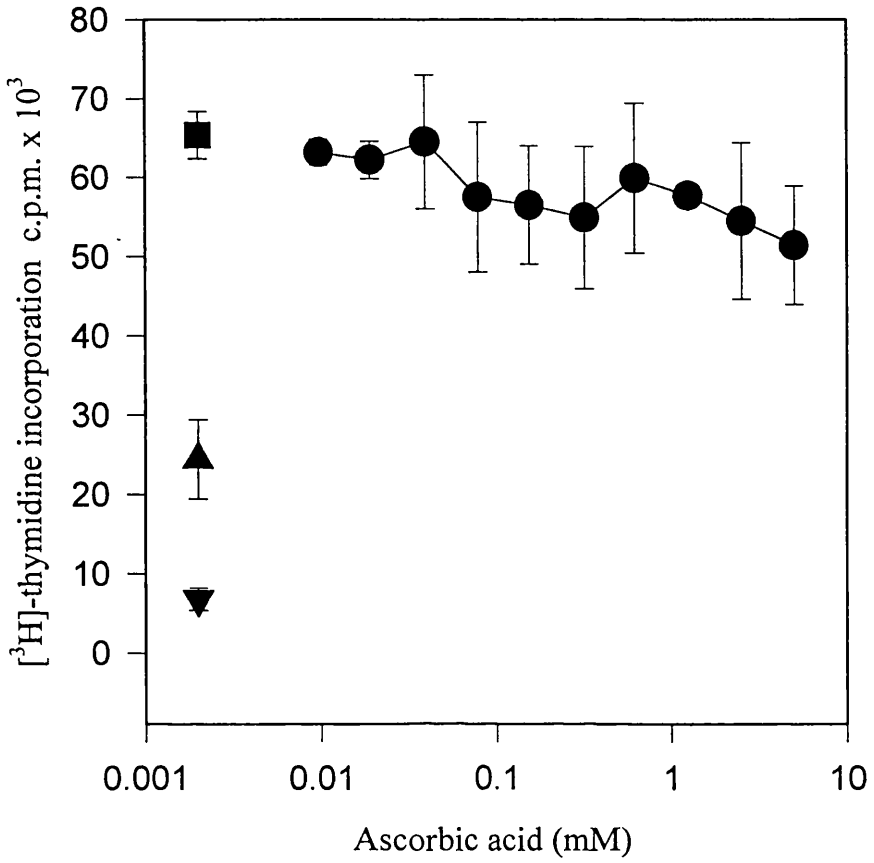


Figure 5.11
The effect of ascorbic acid on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of ascorbic acid on proliferation stimulated with anti-CD28 mAb 15E9, 250ng/ml, plus anti-CD3, 0.1μg/ml. PBMC were incubated with ascorbic acid at the concentrations shown. Anti-CD28 and anti-CD3 were added simultaneously and the cells were incubated for 48 hours prior to the measurement of thymidine incorporation.▼ anti-CD28, 250ng/ml, alone; ▲ anti-CD3, 0.1μg/ml alone; ■ anti-CD28 + anti-CD3; ● anti-CD28 + anti-CD3 + ascorbic acid. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments.

5.12 Effect of anti-CD3 on anti-CD28 mAb 15E9-induced free radical production.

Figure 5.12 shows that the combination of anti-CD28 mAb15E9 and anti-CD3 had an additive effect, but not a synergistic one, for ROS production. The maximum mean florescence change stimulated by a combination of the two antibodies was 20% larger than the ROS signal produced by either antibody alone.

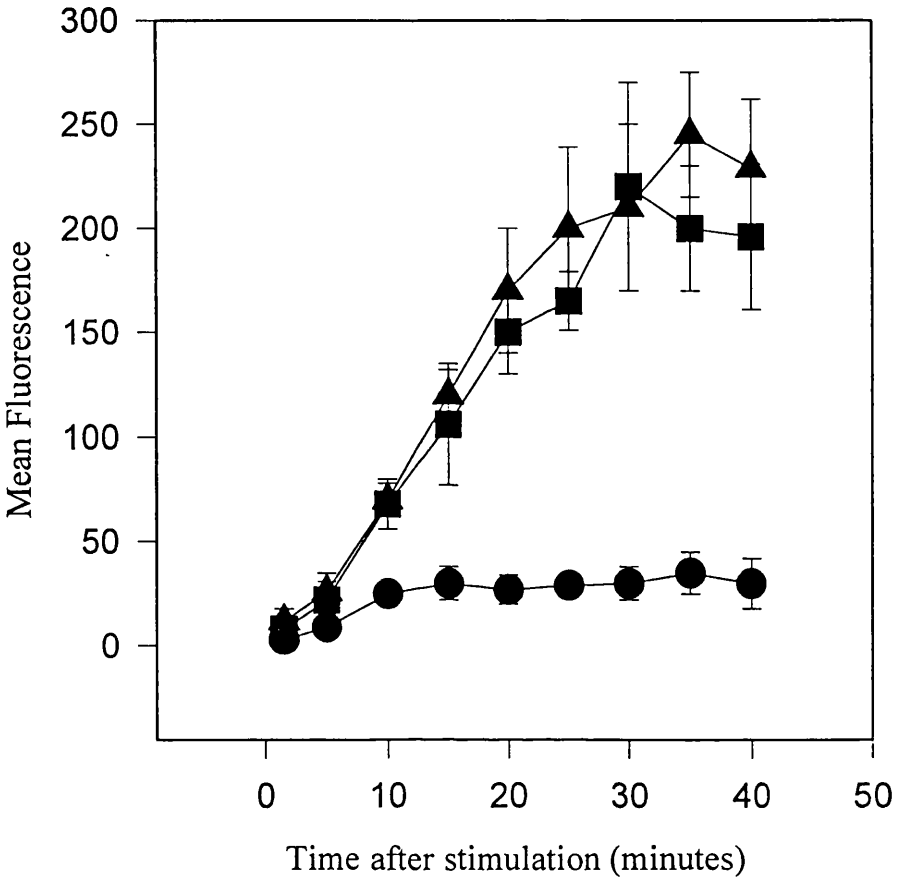


Figure 5.12
The influence of anti-CD28 on the DCF fluorescence from anti-CD3-stimulated PBMC.
The effect of anti-CD28 mAb 15E9, 250ng/ml, on ROS production by the lymphocyte population from PBMC, stimulated with anti-CD3, 0.1μg/ml. Cells were loaded with DCFH-DA, 10μM, 15 minutes before the addition of stimuli: ■ anti-CD28, 250ng/ml, alone; ● anti-CD3, 0.1μg/ml, alone; ▲ anti-CD28 + anti-CD3. Mean fluorescence data was collected every 5 minutes and subtracted from parallel cultures in the absence of anti-CD3 and anti-CD28. The results show the mean ± s.e.m. from three separate experiments.

Discussion.

The aim of the experiments in this chapter was to assess the role of ROS in the T cell activation pathway stimulated by CD28 monoclonal antibodies.

Monoclonal antibodies have been shown to induce human T cell activation via different cell membrane receptors. These T cell surface molecules can be divided into two categories: (i) the T cell antigen receptor associated with the non-polymorphic CD3 antigen; (ii) T cell molecules not linked to the T cell receptor-CD3 complex, such as CD28 and CD2. Stimulation of T cells with these antibodies allows the study of different and "alternative" pathways of activation. The results from this chapter were obtained using two types of the CD28 monoclonal antibody, 913.12 and 15E9.

CD28 is a cell surface homodimeric 41kDa molecule, present on most mature T cells. Much evidence exists suggesting a role for CD28 as a co-receptor in transmembrane signalling during T cell activation. Nevertheless, the nature of the biochemical signalling molecules delivered following CD28 binding are still controversial. The experimental work performed using anti-CD28 mAb 913.12 will be discussed first.

The results presented in Figures 5.1 and 5.2 showed that in the absence of a second signal, anti-CD28 mAb 913.12, 1/800 dilution, had little effect on IL-2 release or proliferation. In line with other reports (Martin *et al.*, 1986), anti-CD28 mAb 913.12, 1/800 dilution, synergised with the phorbol ester, PMA, 0.3nM, for T cell proliferation and IL-2 release. Previous reports with Jurkat cells have demonstrated that CD28 stimulation results in an increase in the intracellular calcium concentration (Weiss *et al.*, 1986). Whether the ability of anti-CD28 mAb 913.12 to synergise with PMA is due to a similar phenomenon to that seen in the synergism observed between the phorbol ester, PMA, and calcium ionophore, A23187, cannot be deduced from these results.

Treatment of PBMC with the antioxidants, desferrioxamine and ascorbic acid inhibited PMA plus anti-CD28 mAb 913.12-induced proliferation in a dose-dependent manner. However, no direct relationship between the effects of these antioxidant compounds on proliferation and IL-2 release could be seen. Desferrioxamine was far less potent with regards to its inhibitory effects on IL-2

release than on proliferation; desferrrioxamine, 0.99μM, was needed to promote 50% inhibition of the control thymidine incorporation, but concentrations greater than 50μM were needed to inhibit IL-2 release to a similar degree. From Figure 5.4, the proliferative actions of desferrioxamine with increasing concentrations appear to occur in a "2-step" manner rather than a steady decline. Initially there was a low sensitivity to the antioxidant (0.024-0.4μM) which was followed by a subsequent sharp decrease in thymidine incorporation. One interpretation of this data would be to suggest that there are two independent ROS-sensitive pathways which co-exist, each with varying sensitivity to the antioxidants used. This is an interesting idea since CD28-mediated activation is thought to trigger at least two independent pathways, one of which is cyclosporin A sensitive (June *et al.,* 1994). Thus, the possibility exists whereby both these pathways are activated by CD28 binding, each with varying ROS-sensitive features. One approach which could be used to examine this idea further would be to determine the effect of CsA on ROS generation: a partial inhibition of the anti-CD28-induced ROS signal would suggest that more than one pathway could be regulating free radical production. Another interpretation of these data is that desferrioxamine is acting by a mechanism additional to its antioxidant properties. Thus, the concentrations of desferrioxamine mediating these two effects would occur over different concentration ranges. Whichever idea, if any, is correct cannot be concluded from the experimental work covered in this chapter.

In contrast to desferrioxamine, the opposite was true for ascorbic acid; the IC_{50} value for IL-2 release was lower (0.61mM) than the IC_{50} value for proliferation (1.59mM). Further studies using a wider range of antioxidants on proliferative and IL-2 assays need to be performed to help understand these data. Unfortunately, unlike desferrioxamine, there is no current data available which may explain an alternative action for ascorbic acid, in addition to its antioxidant properties.

The results presented in Figure 5.3 showed that while anti-CD28 mAb 913.12, 1/800 dilution, synergised with PMA, 0.3nM, for IL-2 release and proliferation, it failed to enhance free radical production. These data are in contrast to those obtained with PMA plus A2387 or anti-CD3 in chapters 3 and 4. These results imply that T cell

activation can occur independent of oxygen radical production. T cell activation is accompanied by increased metabolic activity and oxidative respiration, thus the interaction between anti-CD28 and PMA excludes the possibility that the oxygen free radical production observed with PMA + A23187, and PMA + anti-CD3, was simply a by-product of increased respiratory activity. Of interest in this regard is a recent study which used a similar technique to measure ROS and showed that anti-CD28 activates T cells to produce ROS but fails to synergise with anti-CD3 for oxygen radical production (Los *et al.*, 1995). As for anti-CD28mAb plus PMA, the synergism for proliferation in the study by Los *et al* was independent of an increased ROS signal, supporting the notion that T cell activation can occur independent of oxygen radical production. The exact nature of the CD28 molecule recognised by the antibody used (LCB28) was unclear from the study, but would be of great interest in evaluation of these data.

The second anti-CD28 mAb used in this set of experiments was the monoclonal antibody 15E9. While anti-CD28 mAb 913.12 (purchased from Serotec) was known to react with a 41KDa antigen, the exact nature of the anti-CD28 mAb 15E9 (a gift from Dr. P. Beverly, ICRF) was unclear. To date, only a few studies on the structure-function relationship of the CD28 molecule have been performed, but it is agreed that epitopic heterogeneity of the CD28 molecule influences early and late T cell activation events (Nunes *et al.*, 1993). Interestingly, the data presented in Figure 5.8 showed that whilst anti-CD28 mAb 913.12, 1/800 dilution, alone induced only a small change in the DCF fluorescence (maximum mean fluorescence change of 50 units), anti-CD28 mAb 15E9 was able to generate a much larger ROS signal (maximum mean fluorescence change of 220 units) without the apparent need of a second signal. This effect was not related to the concentration of anti-CD28 mAb 913.12, since increasing the concentration of the antibody failed to increase the DCF fluorescence. It would be interesting to speculate from these results that anti-CD28 mAb 913.12 recognises a region on the CD28 molecule not directly involved in ROS generation.

As with anti-CD28 mAb 913.12, anti-CD28 mAb 15E9, 250ng/ml, alone had little effect on proliferation (IL-2 data using anti-CD28 mAb 15E9 was not determined in these studies), but in the presence of a second "signal" (anti-CD3, 0.1μg/ml), it potentiated thymidine incorporation. It is well documented that to initiate sufficient production of IL-2 for T cell entry into the S phase of the cell cycle, there is a requirement for an "accessory" signal. This second signal is mostly derived from contact with an antigen-presenting cell, as supported by the observation that purified T cells do not produce IL-2 in response to anti-T cell receptor antibodies (Jenkins and Johnson, 1993). The results in this chapter are consistent with the 2 signal hypothesis for T cell activation originally proposed by Lafferty and co-workers (1970). The CD28 cell surface molecule is a receptor capable of generating a co-stimulatory signal which can act in concert with TCR-mediated activation to allow full activation. The results here, in accordance with others, have shown that ligation of CD28 alone has little effect on T cell activation, but it can act in synergy with other stimuli to induce activation, ie. PMA (Ledbetter *et al.*, 1991) and anti-CD3 (Barajo *et al.*, 1989).

The nature of the co-stimulatory signal provided by CD28 is thought to be biochemically distinct from those generated by the TCR. Indeed, an increase in anti-CD28 mAb 15E9-induced intracellular calcium is an unlikely explanation for its action with anti-CD3; anti-CD3 alone induces an increase in intracellular calcium concentration (O'Flynn *et al.*, 1985). In support of this suggestion, Nunes *et al.*, (1993) reported differences in calcium fluxes between different anti-CD28 mAbs, but the exact classification of the calcium-inducing anti-CD28 mAbs was unclear. Further studies directed towards identifying the epitope recognised by anti-CD28 mAb 913.12 and 15E9 would help to clarify this point.

To examine the role of the anti-CD28 mAb 15E9-induced ROS signal in the proliferative response stimulated by anti-CD3 plus anti-CD28 mAb 15E9, the antioxidants desferrioxamine and ascorbic acid were used. The results presented in Figure 5.10 showed that whilst desferrioxamine, 0.024-50μM, inhibited proliferation dose-dependently, it was less potent in comparison to its effect on other activation models. For example, the IC_{50} value of desferrioxamine for proliferation stimulated

by anti-CD3 + anti-CD28 mAb 15E9 was 2.64μM; for PMA + anti-CD28 mAb 913,12 was 0.99μM; for PMA + A23187 was 0.45μM (chapter 3). Ascorbic acid, 0.002-5mM, produced no significant change in thymidine incorporated by cells relative to the control cells stimulated with anti-CD28 mAb 15E9 plus anti-CD3 without the antioxidant.

It is important to establish the validity of the results presented here given that only two antioxidants were tested on one lymphocyte function, ie the proliferative response. Although the use of desferrioxamine and ascorbic acid does not invalidate these results, it is not possible to conclude the exact importance of ROS in the synergistic pathways between anti-CD28 and anti-CD3 for proliferation, or determine whether the nature of the generated radical species, stimulated by anti-CD28 mAb 913.12 and 15E9, differs.

To further the understanding of ROS involvement in the CD28 pathway activated by mAb 15E9 and 913.12, the following questions still need to be answered:

(i) what are the effects of these antioxidants (desferrioxamine and ascorbic acid) plus a wider range of antioxidants with differing modes of action, such as DMSO and vitamin E, on the release of IL-2? This is an important parameter of study since it helps to assess the involvement of ROS in the steps which occur early in T cell activation. Maximum levels of IL-2 and IL-2 receptor expression are reached by about 4 hours after antigen activation (Ullman *et al.,* 1990).

(ii) What are the effects of a series of antioxidant compounds on the ROS signal induced by anti-CD28 mAb 913.12 and 15E9? In this context, the effects of these antioxidants on proliferation and IL-2 assays also needs to be addressed and compared to their actions on free radical formation.

In conclusion, CD28 ligation potentiates the proliferative response induced by PMA and anti-CD3, which can occur independently of ROS generation. The results presented in this chapter also suggest that different anti-CD28 mAbs may differ in their ability to generate a ROS signal.

CHAPTER 6.

STIMULATION OF PURIFIED T CELLS WITH A PHORBOL ESTER AND ANTI-CD3.

Introduction.

Previous experiments (chapters 3, 4, 5) were performed with a mixed cell population, ie. lymphocytes plus accessory cells. Although the oxygen radical signal was being measured in the lymphocyte population of PBMC only, it was important to eliminate the possibility that a phagocyte contamination of the gated lymphocyte population was responsible for the observed oxidative phenomena. The experiments in this chapter were designed to determine whether the presence of other cell types, especially monocytes, were needed to allow T cells to generate ROS.

The purified T cell population, isolated from PBMC using the dynabead method described in chapter 2, was 93-96% CD3+. Thus, the work presented in this chapter measured ROS generated in activated T lymphocytes in the absence of accessory cells.

The experiments performed using a mixed cell population (PBMC) stimulated with a phorbol ester, PMA, and anti-CD3 (chapter 4) were repeated with purified T cells.

Further studies were done to assess whether immobilization of anti-CD3 through monocyte Fc receptors was an essential requirement for T cell ROS formation. A rabbit anti-mouse immunoglobulin was used to cross-link CD3 molecules without Fc receptors, according to the methods described in section 2.12.

6.1 Effect of anti-CD3 on PMA-induced proliferation.

Figure 6.1 shows that anti-CD3, 0.1μg/ml, potentiated the effect of PMA, 0.3nM, in inducing proliferation. There was a 90% increase in thymidine incorporation relative to either stimulus alone.

In comparison to a PBMC population, anti-CD3 alone induced a much smaller proliferative response by purified T cells; the thymidine incorporation by PBMC and purified T cells was 22,000 and 2000 cpm respectively. The lack of a proliferative response by anti-CD3-induced purified T cells confirms the absence of any accessory-derived signals.

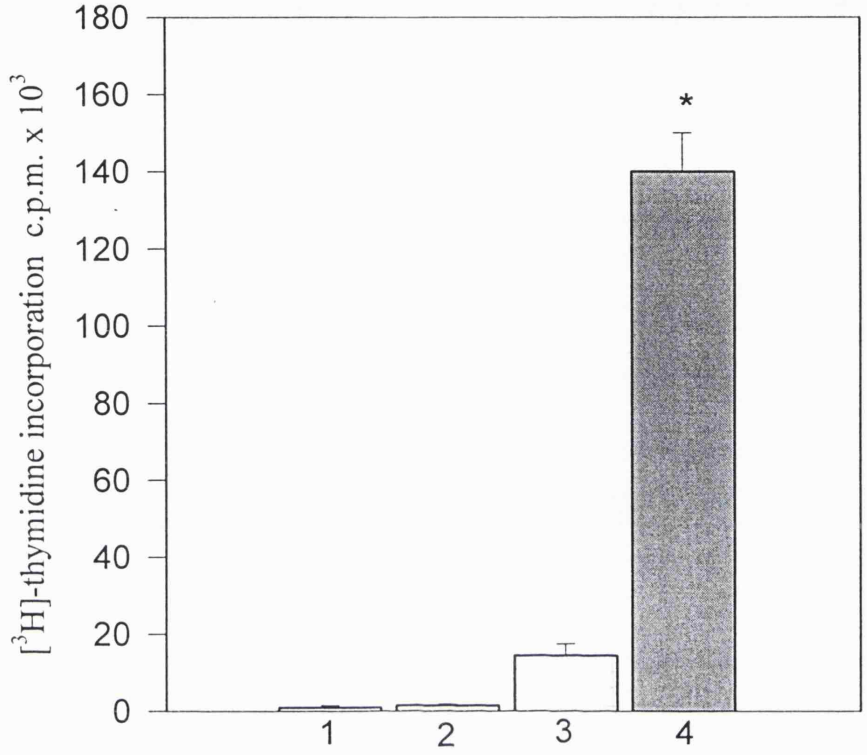


Figure 6.1
The influence of anti-CD3 on the PMA-induced thymidine incorporation by purified T cells.
Effect of anti-CD3, 0.1μg/ml, on proliferation of purified T cells stimulated with PMA, 0.3nM. Cells were treated with: (1) medium alone; (2) anti-CD3, 0.1μg/ml, alone; (3) PMA, 0.3nM, alone; (4) PMA + anti-CD3. After 32 hours, cells were pulsed with [^{3}H]-thymidine and incubated for a further 16 hours. Each bar represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * denotes that the data is significantly different ($p<0.05$) to either stimulus alone.

6.2 Effect of anti-CD3 on PMA-induced free radical production.

In contrast to a mixed cell population, Figure 6.2 shows that anti-CD3, 0.1μg/ml, failed to potentiate the PMA-induced ROS signal.

PMA, 0.3nM, alone induced a similar magnitude of increase in the DCF fluorescence as was seen using PBMC; maximum mean fluorescence change was 650 units. The profile of the effect of PMA on the increase in DCF fluorescence by purified T cells and by the lymphocyte population from PBMC was no different. Both started with a delay of approximately 10 minutes and were linear over the next 25-30 minutes.

The anti-CD3-induced ROS signal was completely absent; the maximum mean fluorescence change by PBMC was 25 units.

6.3 Effect of cross-linking CD3 on free radical production by purified T cells.

An important parameter in T cell receptor-mediated activation is the aggregation of several T cell receptors in close proximity. Monocyte Fc receptors are thought to favour such aggregation (cross-linking). To determine if the failure of anti-CD3 to synergise with PMA for ROS generation was simply due to the lack of CD3 cross-linking, a rabbit anti-mouse immunoglobulin was used. The rabbit anti-mouse immunoglobulin provides a frame for binding and immobilizing the Fc part of the anti-T cell monoclonal antibodies.

Three different protocols were used to cross-link CD3 molecules, as described in chapter 2. Similar results were obtained with all three methods.

Figure 6.3 shows the results from using protocol 1, ie. "pre-formed complexes". Here the rabbit anti-mouse immunoglobulin was mixed with anti-CD3 before addition to the cells. The cells were incubated with the "mixture" for 15 minutes prior to the addition of the free radical sensitive dye, DCFH-DA. The cells were then stimulated with PMA, 0.3nM, in the normal way.

The rabbit anti-mouse immunoglobulin failed to potentiate the anti-CD3-induced ROS signal. Cross-linking did not allow anti-CD3 to potentiate the PMA-induced free radical production, thus implying that the failure of anti-CD3 to increase ROS production stimulated by PMA was not due to a lack of CD3 cross-linking.

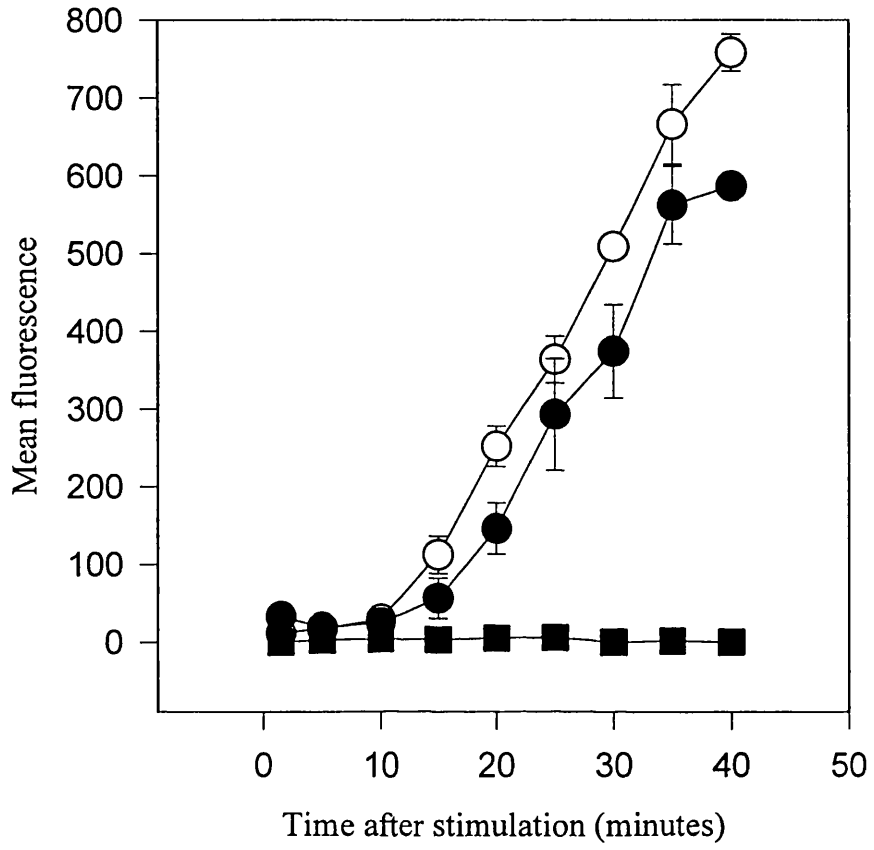


Figure 6.2
The influence of anti-CD3 on the DCF fluorescence from PMA-stimulated T cells.
Effect of anti-CD3, 0.1μg/ml, on ROS production by purified T cells stimulated with PMA, 0.3nM. Cells were loaded with DCFH-DA, 10μM, 15 minutes before stimulation with: ■ anti-CD3, 0.1μg/ml, alone; ○ PMA, 0.3nM, alone; ● anti-CD3 + PMA. Fluorescence data was collected every 5 minutes and subtracted from parallel cultures in the absence of PMA and anti-CD3. The data are the mean ± s.e.m. from three separate experiments.

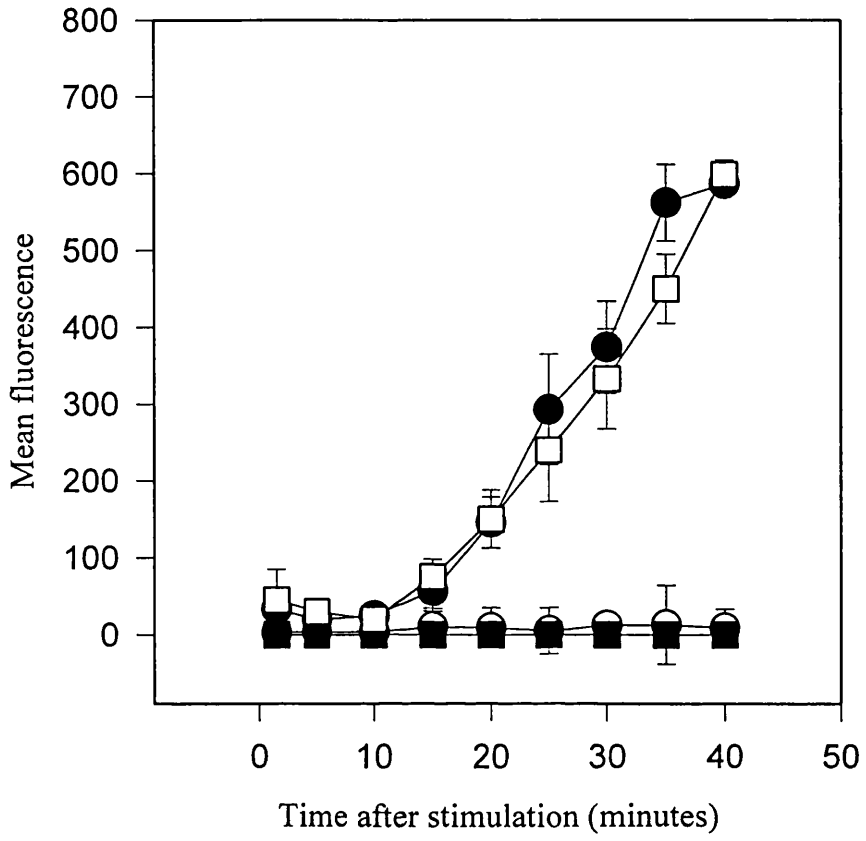


Figure 6.3
The effect of cross-linking CD3 on the DCF fluorescence of T cells.
The effect of crosslinking anti-CD3 with a rabbit anti-mouse immunoglobulin on the DCF fluorescence of T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. A mixture of a rabbit anti-mouse immunoglobulin (final concentration 1/100) and anti-CD3 (final concentration 0.1μg/ml) was prepared at 4°C. Cells were loaded with DCFH-DA, 10μM, 15 minutes before stimulation with: ■ anti-CD3, 0.1μg/ml alone; ○ anti-CD3 + immunoglobulin; ● PMA, 0.3nM, alone; ❑ PMA + anti-CD3 + immunoglobulin. Fluorescence data was collected every 5 minutes and subtracted from parallel cultures in the absence of PMA, anti-CD3 and immunoglobulin. The data are the mean ± s.e.m. from three separate experiments.

Effect of antioxidants on the proliferative response of purified T cells stimulated by PMA in synergy with anti-CD3.

To further investigate the role of ROS-mediated signalling in a purified T cell sample, the series of experiments performed with the antioxidants in chapter 4 were repeated using purified T cells; desferrioxamine, ascorbic acid, DMSO, vitamin E and NAC.
Cell viability was tested by trypan blue exclusion and was unaffected by the antioxidants at the highest concentration used.
Control samples were stimulated with anti-CD3, 0.1μg/ml, plus PMA, 0.3nM, without antioxidant.

6.4 Effect of desferrioxamine on proliferation.

Figure 6.4 shows that desferrioxamine, 0.024-50μM, produced a dose-dependent inhibition of proliferation by purified T cells. This effect ranged from about 14% below control at 0.024μM to 100% inhibition of the proliferative response at 50μM. The concentration of desferrioxamine needed to promote 50% inhibition of the control thymidine incorporation was 0.22 ± 0.71μM.

6.5 Effect of ascorbic acid on proliferation.

Figure 6.5 shows that ascorbic acid, 0.002-5mM, dose-dependently inhibited PMA + anti-CD3-induced thymidine incorporation. This effect ranged from about 15% below control at 0.002mM to 100% inhibition of proliferation at 5mM. The concentration of ascorbic acid needed to inhibit proliferation by 50% of control was 0.077 ± 0.09mM.

6.6 Effect of DMSO on proliferation.

Figure 6.6 shows that DMSO at concentrations from 0.19 to 200mM produced a dose-dependent decrease in the amount of thymidine incorporated by purified T cells. This effect ranged from about 25% below control at 0.19mM to 100% inhibition of control value at 200mM. The concentration of DMSO needed to promote 50% inhibition of the control thymidine incorporation was 74.9 ± 7.0 mM.

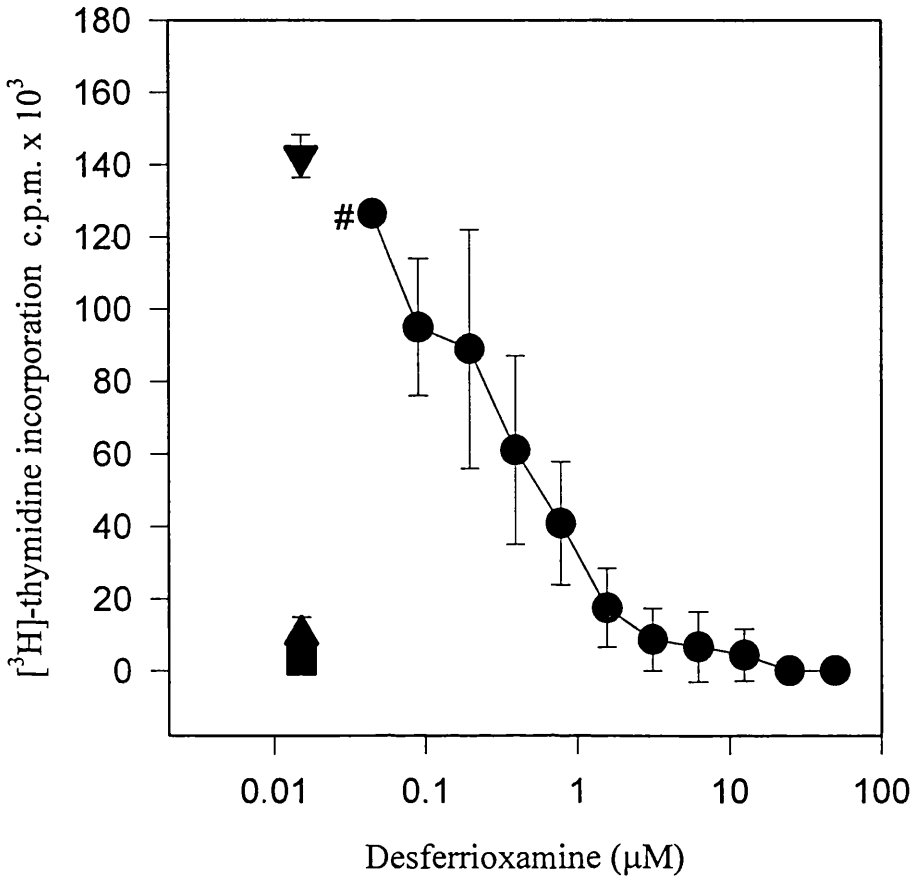


Figure 6.4

The effect of desferrioxamine on thymidine incorporation by purified T cells.

The concentration-response relationship for the effect of desferrioxamine on proliferation of purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were incubated with desferrioxamine at the concentrations shown. PMA and anti-CD3 were added simultaneously. After 32 hours, cells were pulsed with [^{3}H]-thymidine and incubated for a further 16 hours. ▲ PMA, 0.3nM, alone; ■ anti-CD3, 0.1μg/ml, alone; ▼ PMA + anti-CD3; ● PMA + anti-CD3 + desferrioxamine. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. # indicates that a point is not significantly different ($p>0.05$) from control values in the absence of desferrioxamine, using the Student's t test. The IC_{50} of desferrioxamine was 0.22 ± 0.71μM.

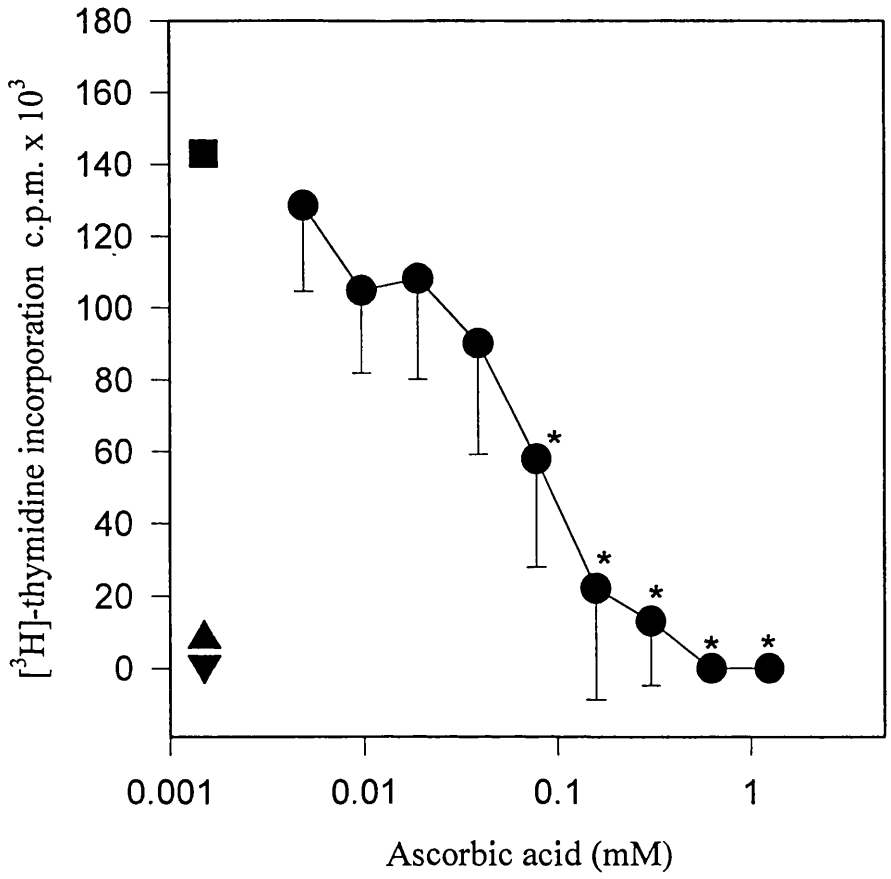


Figure 6.5

The effect of ascorbic acid on thymidine incorporation by purified T cells.

The concentration-response relationship for the effect of ascorbic acid on proliferation stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were incubated with ascorbic acid at the concentrations shown. PMA and anti-CD3 were added simultaneously. A pulse of [^{3}H]-thymidine was added for the last 16 hours of the 48 hour incubation period. ▲ anti-CD3, 0.1μg/ml, alone; ▼ PMA, 0.3nM, alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + ascorbic acid. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of ascorbic acid, using the Student's t test. The IC_{50} of ascorbic acid was 0.077 ± 0.09mM.

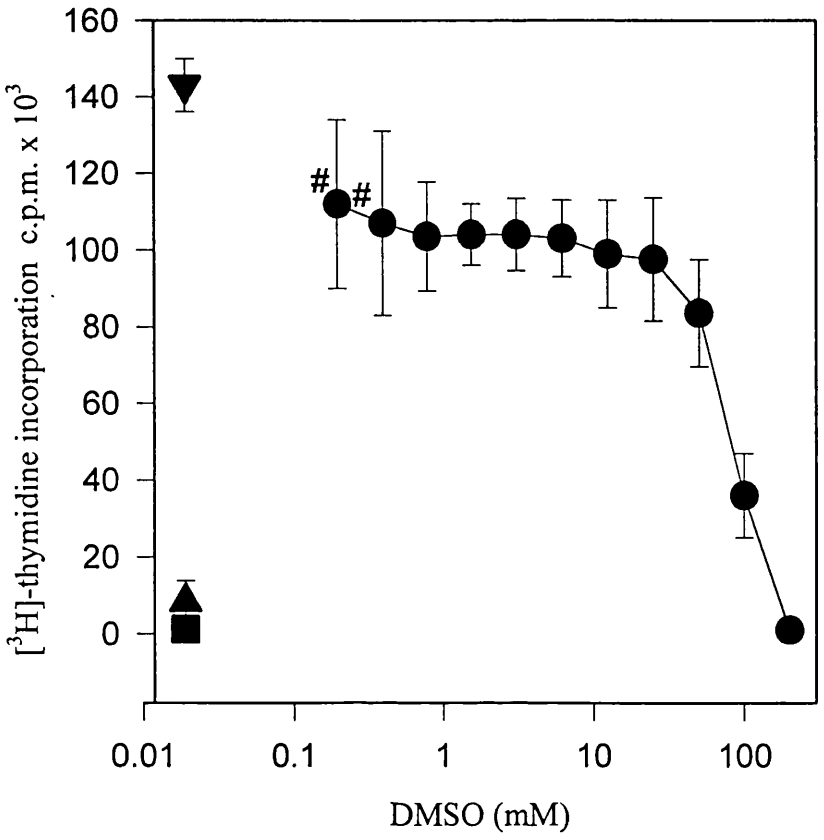


Figure 6.6
The effect of DMSO on thymidine incorporation by purified T cells.
The concentration-response relationship for the effect of DMSO on proliferation of purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were treated with: ▲ PMA, 0.3nM, alone; ■ anti-CD3, 0.1μg/ml, alone; ▼ PMA + anti-CD3; ● PMA + anti-CD3 + DMSO, at the concentrations shown. [^{3}H]-thymidine was added for the last 16 hours of the 48 hour culture. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. # indicates that a point is not significantly different ($p>0.05$) from control values in the absence of DMSO, using the Student's t test. The IC_{50} of DMSO was 74.9 ± 7.0 mM.

6.7 Effect of vitamin E on proliferation.

Figure 6.7 shows that vitamin E, 0.0019-3mM, had no significant effect on proliferation stimulated by PMA, 0.3nM, plus anti-CD3, 0.1μg/ml.

6.8 Effect of NAC on proliferation.

Figure 6.8 shows that NAC had a "triphasic" dose-dependent action on purified T cell proliferation. Concentrations of NAC between 0.0488-0.39mM dose-dependently inhibited proliferation ("first phase"). This effect ranged from about 29% below control at 0.0488mM to about 54% below control at 0.39mM. NAC, at concentrations greater than 0.39mM to 3.12mM, had effects which were not statistically significantly different from those of cells stimulated without NAC ("second phase"). At concentrations which were greater than 3.12mM, NAC caused maximum inhibition, with about 95% inhibition of the control proliferative response at 10mM ("third phase").

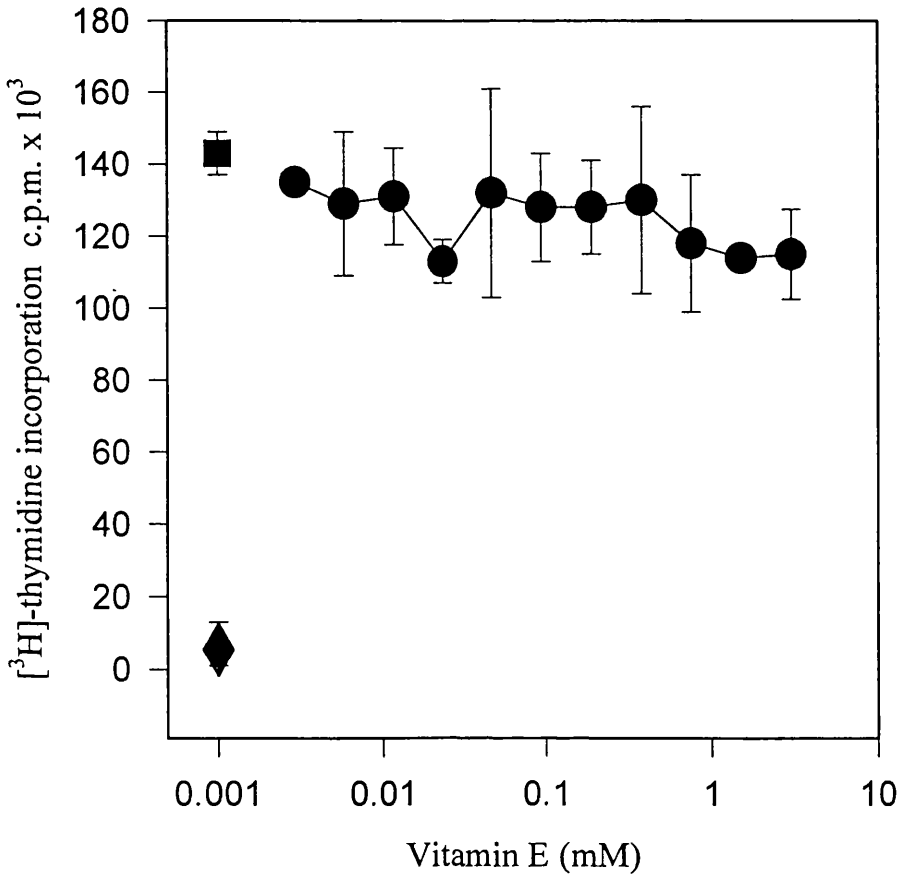


Figure 6.7
The effect of vitamin E on thymidine incorporation by purified T cells.
The concentration-response relationship for the effect of vitamin E on proliferation of purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were incubated with vitamin E at the concentrations shown. PMA and anti-CD3 were added simultaneously and the cells were cultured for 48 hours prior to harvesting. ▲ PMA, 0.3nM, alone; ▼ anti-CD3, 0.1μg/ml, alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + vitamin E. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments.

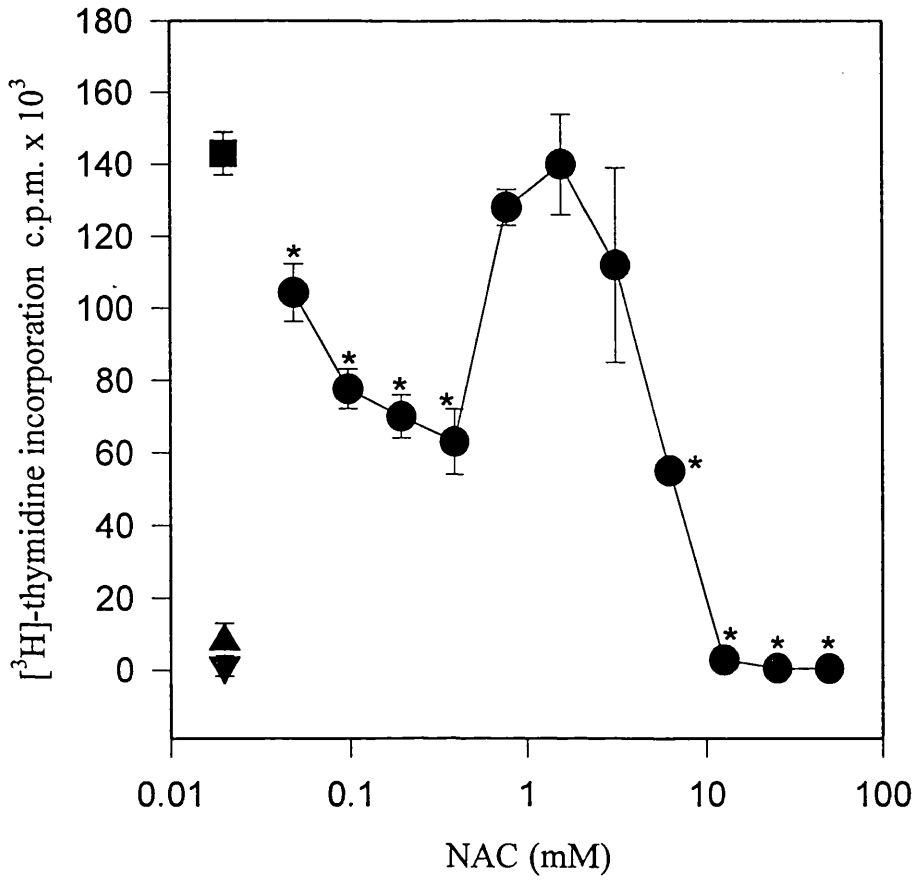


Figure 6.8
The effect of NAC on thymidine incorporation by purified T cells.
The concentration-response relationship for the effect of NAC on proliferation of purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were incubated with NAC at the concentrations shown. PMA and anti-CD3 were added simultaneously and the cells were incubated for 48 hours prior to the measurement of thymidine incorporation. ▲ PMA, 0.3nM, alone; ▼ anti-CD3, 0.1μg/ml, alone; ■ PMA + anti-CD3; ● PMA + anti-CD3 + NAC. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. * indicates that a point is significantly different ($p<0.05$) from control values in the absence of NAC.

Effect of antioxidants on free radical production stimulated by PMA plus anti-CD3.

The antioxidants were added to a suspension of purified T cells 15 minutes prior to the addition of the fluorescent dye. Cells were incubated for a further 15 minutes, before the addition of PMA + anti-CD3.

The concentration of antioxidant chosen inhibited the proliferative response by more than 80%. The concentration of NAC was chosen from the "first" dose-dependent inhibitory phase.

6.9 Effect of desferrioxamine and DMSO on ROS formation by purified T cells.

Figure 6.9 shows that desferrioxamine, 10μM, and DMSO, 100mM, did not significantly decrease ($p>0.05$) the ROS signal stimulated by PMA + anti-CD3.

This is in contrast to the effects of desferrioxamine and DMSO on the ROS signal produced by PBMC. Both these antioxidants are thought to act primarily on the hydroxyl radical, thus implying the nature of the ROS signal, in the presence and absence of accessory cells, differs.

6.10 Effect of NAC and vitamin E on ROS formation by purified T cells.

Figure 6.10 shows that NAC, 0.1mM, and vitamin E, 1.5mM, had no significant effect ($p>0.05$) on the DCF fluorescence of purified T cells, stimulated by PMA + anti-CD3.

6.11 Effect of ascorbic acid on ROS formation by purified T cells.

Figure 6.11 shows that ascorbic acid, 0.5mM, at a concentration which inhibited proliferation by more than 90% also inhibited ROS production by a similar degree. This inhibitory effect was statistically significantly different ($p<0.05$) from cells stimulated with PMA + anti-CD3 without ascorbic acid.

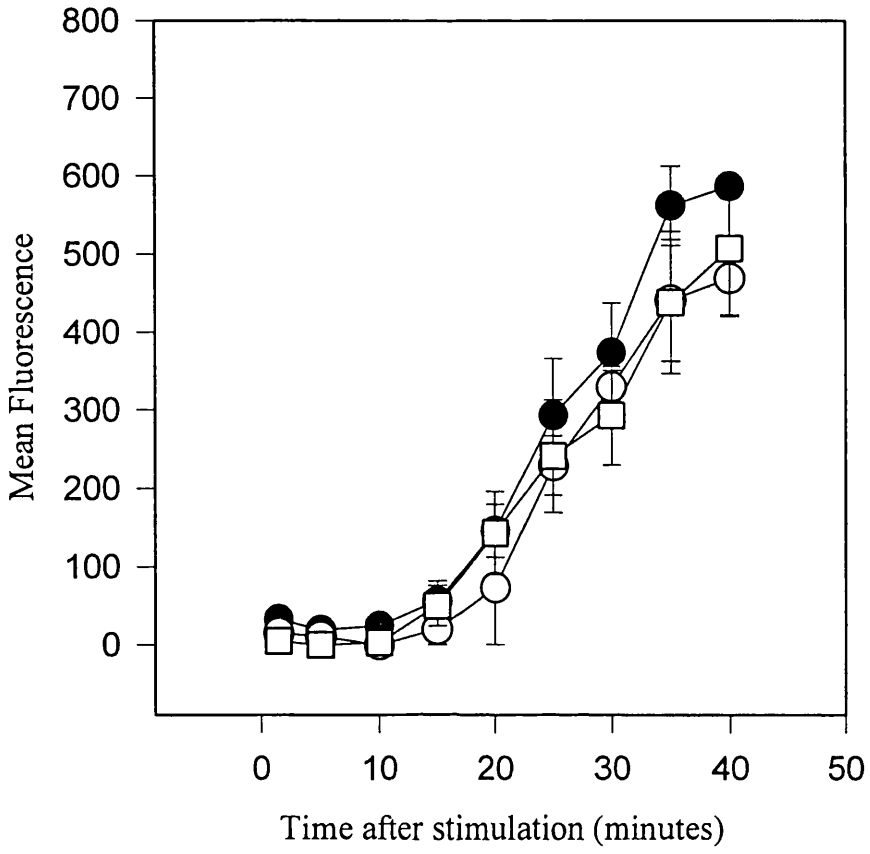


Figure 6.9
The effect of DMSO and desferrioxamine on the DCF fluorescence of T cells.
The effect of DMSO, 100mM, and desferrioxamine, 10μM, on ROS production by purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were pretreated 30 minutes prior to the addition of stimulus with: ● medium; ❑ desferrioxamine, 10μM; ○ DMSO, 100mM. Mean fluorescence readings were taken every 5 minutes for 40 minutes. The data are the mean ± s.e.m. from three separate experiments.

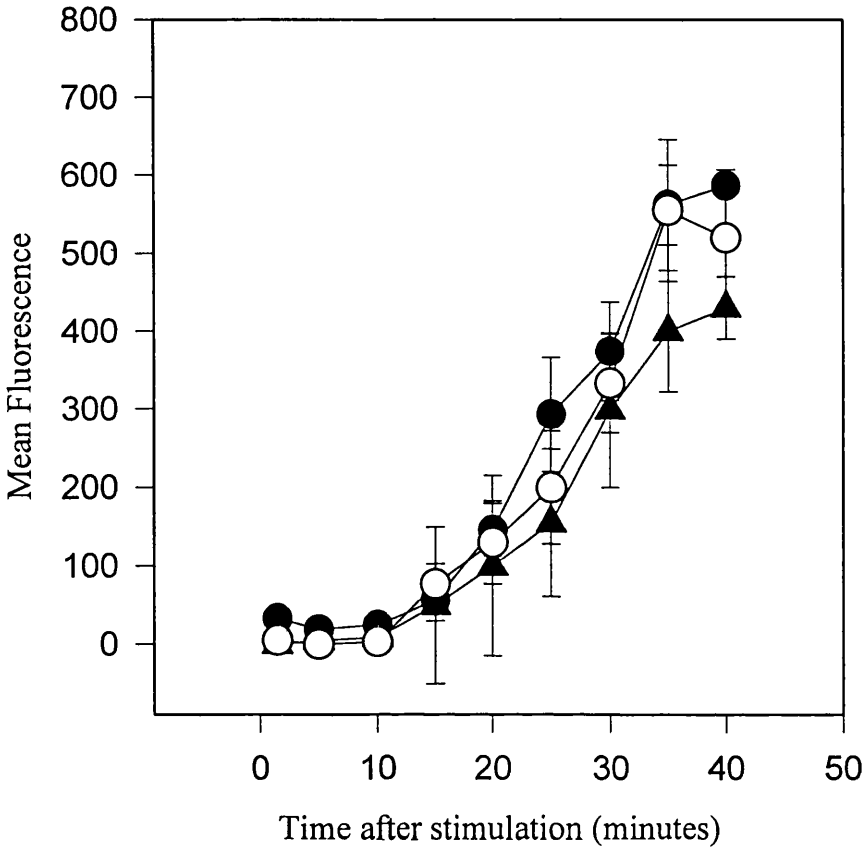


Figure 6.10
The effect of NAC and vitamin E on the DCF fluorescence of T cells.
The effect of NAC, 0.1mM, and vitamin E, 1.5mM, on ROS production by purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. Cells were pretreated 30 minutes prior to the addition of stimulus with: ● medium; ▲ NAC, 0.1mM; ○ vitamin E, 1.5mM. Mean fluorescence data was collected every 5 minutes and subtracted from parallel cultures in the absence of PMA and anti-CD3. The data are the mean ± s.e.m. from three separate experiments.

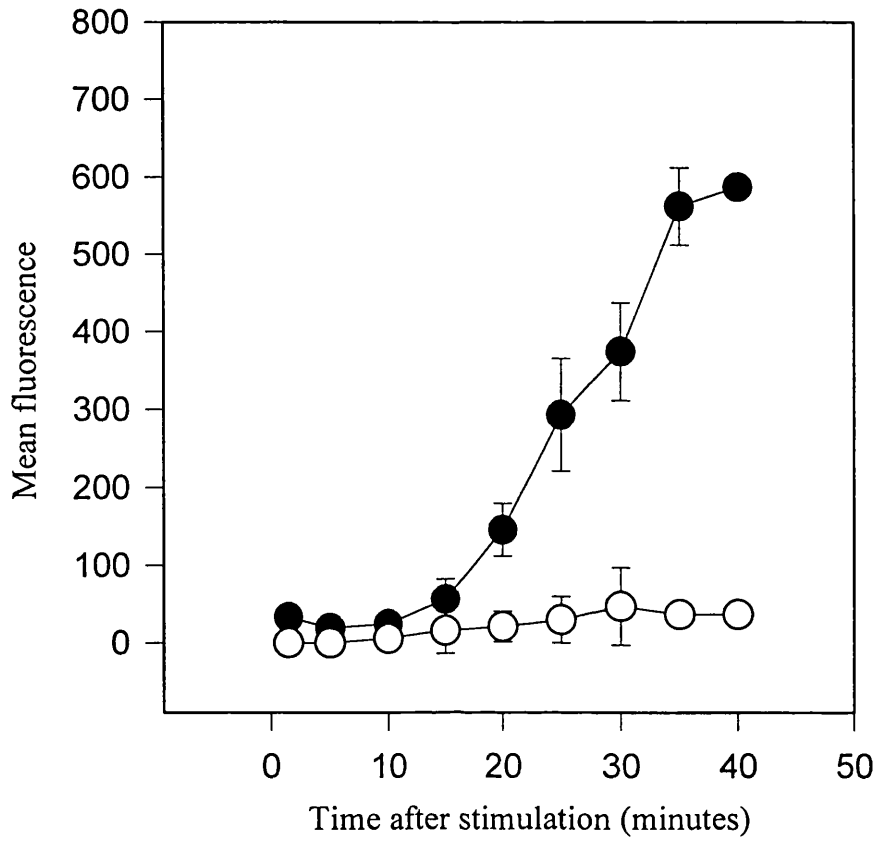


Figure 6.11
The effect of ascorbic acid on the DCF fluorescence of purified T cells.
The effect of ascorbic acid, 0.5mM, on ROS production by purified T cells stimulated with PMA, 0.3nM, plus anti-CD3, 0.1μg/ml. 30 minutes before the addition of stimulus, cells were treated with: ● medium; ○ ascorbic acid, 0.5mM. The data are the mean ± s.e.m. from three separate experiments. 2-way ANOVAs were used to statistically analyse the data. The p value obtained for no ascorbic acid vs 0.5mM ascorbic acid was 0.0012.

Overall summary: Chapters 3-6.

The effective concentration of antioxidant for 50% inhibition of proliferation, IL-2 release and DCF fluorescence stimulated by PMA + anti-CD3 (with/out accessory cells) or PMA +A23187, or PMA +anti-CD28 mAb 913.12, or anti-CD3 +anti-CD28 mAb 15E9.

Table 6.1 shows the concentration of antioxidant needed to promote 50% inhibition of the response relative to its control value (cells stimulated with the corresponding activation model without antioxidant).

To ascertain whether there was any relationship between the concentration of antioxidant promoting 50% inhibition of the control (IC_{50}) proliferation, IL-2 release and DCF fluorescence, the results shown in the table are from all the different activation models used in this study.

With all modes of stimulation, the IC_{50} value for proliferation and IL-2 release were a lot higher than for DCF fluorescence.

The concentration of antioxidant needed to inhibit proliferation induced by PMA + anti-CD3 by 50% of control in a mixed cell population were higher than that needed in purified T cell, for all the antioxidants used.

The IC_{50} values for proliferation stimulated by PMA + A23187 and PMA + anti-CD28 mAb 913.12 were similar in magnitude.

Although the antioxidants used in this work produced significant inhibition of lymphocyte functions stimulated by a variety of stimuli, there was no direct relationship between the IC_{50} values for proliferation, IL-2 release and ROS generation, within any one combination of stimuli tested.

Activation Model	Antioxidant	Thymidine incorporation	Il-2 release	DCF Fluorescence
PMA + A23187 (PBMC)	Desferrioxamine (μM)	0.45 ± 0.21	#	~0.01
	Ascorbic acid (mM)	2.97 ± 0.13	#	~5 x 10^{-4}
PMA + anti-CD3 (PBMC)	Desferrioxamine (μM)	1.52 ± 0.13	10.98 ±1.92	~0.01
	Ascorbic acid (mM)	1.19 ± 0.46	1.03 ± 0.13	~5 x 10^{-4}
	DMSO (mM)	162.53 ± 21.36	197.6 ± 5.54	~100
	Vitamin E (mM)	0.60 ± 0.05	0.79 ± 0.04	#
	NAC (mM)	2.50 ± 0.13	=	#
PMA + anti-CD28 (PBMC)	Desferrioxamine (μM)	0.99 ± 0.09	#	=
	Ascorbic acid (mM)	1.59 ± 0.12	0.61 ± 0.03	=
anti-CD28 + anti-CD3 (PBMC)	Desferrioxamine (μM)	2.64 ± 0.27	=	=
	Ascorbic acid (mM)	#	=	=
PMA+ anti-CD3 (purified T cells)	Desferrioxamine (μM)	0.22 ± 0.71	=	#
	Ascorbic acid (mM)	0.08 ± 0.09	=	~ 0.1
	DMSO (mM)	74.97 ± 7.01	=	#
	Vitamin E (mM)	#	=	#
	NAC (mM)	~ 0.39	=	#

Table 6.1 Overall summary: Chapters 3-6
The effective concentration of antioxidant for 50% inhibition of proliferation, Il-2 release and DCF fluorescence.
Data are presented as the concentration of antioxidant needed to inhibit the response by 50% of its control value, in the absence of antioxidant. # indicates that the antioxidant had no significant effect on the response, over the range of concentrations tested. Where ~ appears next to a value, it is an approximation. = denotes that no value is obtainable. The results are from atleast three separate experiments. IC_{50} values were calculated from a Hill plot.

Discussion.

The main objective of this chapter's experiments was to determine whether the presence of any other cell types, particularly monocytes, was an essential requirement in allowing T cells to generate oxygen free radicals. Previous experiments, in chapter 3, were performed with catalase to establish an intracellular site of ROS formation.

The monocyte population produced higher levels of DCF fluorescence than the lymphocyte population from stimulated PBMC samples (data not shown). This was to be expected as monocytes are phagocytic cells and respond to stimulation by undergoing a respiratory burst, generating hydrogen peroxide and superoxide anion radicals (Babior, 1988). By gating separate cell populations, analysis of the fluorescence derived from the lymphocytes and monocytes could be measured separately. The oxygen radical signal measured in chapters 3, 4 and 5 was from the lymphocyte population of PBMC only. However, it was important to eliminate the possibility that a phagocyte contamination of the gated lymphocyte population was responsible for the observed oxidative phenomena. The experiments conducted in this chapter used purified T cell populations isolated from PBMC, using the dynabead method described in chapter 2 (Methods). The lymphocyte population after purification was 93-96% CD3+ (the lymphocyte population from an unseparated leukocyte population was 76% CD3+). Therefore, the ROS signal being detected was assumed to be from activated T cells in the absence of accessory cells.

Cultures of purified T cells failed to proliferate in response to anti-CD3, 0.1μg/ml, alone. These data confirm the purity of the isolated T cells and suggest that accessory cells provide the second signal needed for proliferation. The fact that anti-CD3 synergised with PMA in PBMC (Figure 4.1) and also in purified T cells (Figure 6.1), suggests that PMA can replace the need for a second accessory cell-dependent signal (IL-2 release from purified T cells stimulated by PMA plus anti-CD3 was not evaluated here). Interaction of anti-CD3 with its receptor results in an increased intracellular calcium concentration, providing one of the two activation signals needed for a full activation response. PKC activation by PMA provides the second. How the increase in calcium and PKC activation result in IL-2 gene regulation is

unclear, and whether it occurs by a mechanism similar to that seen with PMA and A23187 remains to be established.

The results presented in chapter 4 showed that PMA, 0.3nM, alone induced a small ROS signal. The PMA-induced ROS signal in purified T cells was of equal magnitude (Figure 6.2). Hence, these results provide evidence that PMA-activated T cells can produce oxygen free radicals in the absence of other leukocytic components. These results are in disagreement with other published reports. Many studies have been performed using a DCF assay for ROS measurements, but none have shown lymphocytes to increase their fluorescence upon stimulation. Some of these studies (Robinson *et al.,* 1988) only monitored cell fluorescence for the initial 15 minute period, during which time my data showed that there was only a small increase in DCF fluorescence. Others reported that PMA-activated lymphocytes could only generate ROS if they were in the presence of phagocytes (Rabesandratana *et al.,* 1992). In this latter study, the technique used to purify T lymphocytes was different from the one employed in this chapter. It is possible that the use of a gradient technique to separate lymphocytes from other leukocyte components may change the nature of lymphocytes in some way, perhaps altering an essential membrane-component of the ROS generation pathway. It has become apparent that T lymphocytes may possess a membrane-component, cytochrome-b-like, of the NADPH oxidase found in phagocytic cells (Pick and Gabda, 1988). This is discussed further in chapter 8 (general).

Although the addition of anti-CD3 enhanced PMA-induced T cell proliferation (as was the case for PBMC), the results from Figure 6.2 show that the anti-CD3, 0.1μg/ml, failed to synergise with PMA for ROS formation in purified T cells. Furthermore, even the small increase in DCF fluorescence stimulated by anti-CD3, 0.1μg/ml, in a PMBC sample (20 units) was absent in purified T cells.

An important factor in T cell receptor-mediated activation is the aggregation of several T cell receptors in close proximity. It is thought that immobilisation of anti-CD3 through monocyte Fc receptors favours such aggregation (cross-linking) and is, therefore, an important requirement for T cell stimulation. Since a suspension of

purified T cells does not contain any monocytes, and hence no monocyte Fc receptors, it was important to determine whether the failure of anti-CD3 to generate a ROS signal, or synergise with PMA for potentiation of DCF fluorescence was simply due to a lack of CD3 cross-linking. This question was addressed by using a rabbit anti-mouse immunoglobulin to cross-link anti-CD3, without monocyte Fc receptors. The fact that cross-linking with a rabbit anti-mouse second antibody failed to change the fluorescence reading discards the possibility that lack of ROS generation was due to the absence of CD3 cross-linking via immobilization through monocyte Fc receptors (Figure 6.3).

By examining the action of various antioxidant compounds on the ROS signal generated in purified T cells, the nature of the ROS signal was concluded to be different from that produced by lymphocytes in a mixed population. Whilst desferrioxamine and DMSO inhibited the DCF fluorescence induced by the lymphocyte population of PBMC, neither antioxidant inhibited free radical production by purified T cells. Desferrioxamine is an iron chelator. Since the production of the hydroxyl radical is catalysed by iron, it is probable that it exerts its action primarily on this oxygen radical species. As DMSO is known to be a scavenger of hydroxyl radicals, it would indeed be interesting to speculate that the major species detected in the PBMC ROS signal is the hydroxyl radical. The results presented in Figure 6.11 showed that the ROS signal produced by purified T cells was very effectively blocked by ascorbic acid, 0.5mM (>90% inhibition). Ascorbic acid acts as a potent reducing agent for pre-existing ROS within the cell, and has been shown to react with superoxide anion radicals (Niki, 1991). These results imply that the main species being detected in the ROS signal generated by the purified T cell population is different from that produced by lymphocytes in the presence of accessory cells. It could be that the major radical detected in the PMA-induced ROS signal by PBMC is the hydroxyl radical, while the superoxide anion radical may be more important in the PMA-induced ROS signal in the absence of accessory cells. Further studies need to be done to investigate the possibility that the nature of the radical species generated in the absence and presence of accessory cells is indeed different. It is unfortunate that

as yet there is no technique available for the distinction of radical species in vivo directly. One approach would be to assess the scavenging capacity of dimethylthiourea. Dimethylthiourea does not react with superoxide anions but reacts powerfully with hydroxyl radicals, with a rate constant of approximately $10^{10}M^{-1}s^{-1}$ (Fox *et al.,* 1984). If superoxide anion radicals are the "key-players" in the PMA-induced ROS signal produced by purified T cells, it would be expected that dimethylthiourea would have no significant effect. Interestingly, a recent study has shown that PBMC stimulated with anti-CD3 can generate the hydroxyl radical, but do not produce any hydrogen peroxide or superoxide anion radicals (Whitacre *et al.,* 1992).

DMSO and desferrioxamine had no effect on the ROS signal generated by purified T cells, but both antioxidants were effective in inhibiting the proliferative response (Figures 6.4, 6.6). It may be that these antioxidant compounds are acting at more than one site. In addition, it could be that desferrioxamine is influencing other lymphocyte functions indirectly through its action on iron. Lymphocyte proliferation has been shown to be dependent on iron (Gutteridge *et al.,* 1979), and it may be possible for desferrioxamine to exert its action indirectly via iron-containing enzymes. Alternatively, it could be that although ROS formation in T cells is stimulated by T cell activation, ROS act by influencing more than one pathway, rather than than just acting on specific "oxidative-sensitive receptors". The data imply that the relationship between ROS formation and subsequent T cell activation is not a simple one.

To investigate the relationship between ROS generation and T cell activation further, two other antioxidants were used; vitamin E (α-tocopherol) and N-acetyl cysteine (NAC). The role of vitamin E as a cellular lipid-phase antioxidant is well recognised (Packer and Landvik, 1989). NAC has been shown to scavenge oxidants directly and to increase intracellular glutathione levels; glutathione being a major intracellular redox buffer of oxidative stress (Arnoma *et al.,* 1989).

Vitamin E and NAC inhibited proliferation of PBMC, but not of purified T cells. The contrasting data obtained using these antioxidants on PBMC, and on purified T cells stimulated with PMA in synergy with anti-CD3, provides further support for the

involvement of differing ROS signalling mechanisms in the absence, and presence, of accessory cells. Another point of difference seen with the effects of antioxidants on purified T lymphocytes and PBMC is depicted in Figure 6.8. NAC elicited an unusual "triphasic" dose-dependent action on purified T cells stimulated by PMA plus anti-CD3. Since NAC enhanced CTLL growth in the presence of a fixed concentration of human recombinant IL-2 (as shown in Figure 2.2), the action of NAC could be related directly to its ability to replenish intracellular glutathione. Glutathione has been well documented as an essential compound for mitogenesis (Fidelus *et al.*, 1986; Hamilos *et al.*, 1991). Furthermore, the proliferative capacity of human blood lymphocytes has been reported to be dependent on the cells' intracellular glutathione content (Kavanagh *et al.*, 1990). It is possible therefore, that the increased IL-2 synthesis triggered by NAC (and indirectly glutathione), contributes in part to the unusual effect seen with NAC-treated purified T cells. In support of this, Eylar *et al.*, (1993) reported that NAC could enhance T cell growth in culture and this effect was partly attributed to NACs ability to increase intracellular glutathione. The data here showed that while low NAC concentrations (less than 0.39mM) dose-dependently inhibited proliferation by purified T cells, concentrations greater than 0.39mM, but less than 3.12mM, resulted in an effect not significantly different from the control response without NAC. Concentrations greater than 10mM had an inhibitory action.

By what mechanism NAC contributes exactly to the proliferative response of PMA plus anti-CD3-stimulated purified T cells is still controversial. Transcription factors regulating the IL-2 gene include NFAT-1, AP-1, Oct-1 and NF-κB. Activation of the IL-2 gene requires the co-ordinated efforts of all these factors. While glutathione has been reported to inhibit the activity of NF-κB (Meyer *et al.*, 1994), other transcription factors may be activated by an increase in glutathione levels. It would be interesting to speculate that different ROS are generated in purified T cells compared to the radical species in T cells from a mixed population; their influence in turn on intracellular transcription factors being by different/separate regulatory mechanisms. Furthermore, while one transcription factor could be sensitive to glutathione levels, another may be more sensitive to changes in the ROS milieu. In summary, these data

imply that the regulation of IL-2 transcription and the possible role of glutathione in this, is a complex one.

A further interesting feature of NAC, which may be relevant to its action on purified T cells is its mode of ROS decomposition. NAC reacts powerfully with the hydroxyl radical, moderately with hydrogen peroxide, and poorly with the superoxide anion radical (Aruoma *et al.,* 1989). Thus, if indeed different radicals are generated by lymphocytes in the presence (PBMC) and absence (purified T cells) of accessory cells, NAC would have differing activity on the two types of cell populations. Furthermore, it has been shown that NAC-mediated decomposition of hydrogen peroxide occurs in a biphasic manner (Arroub *et al.,* 1994), with an initial rapid decline in hydrogen peroxide being followed by a slower phase of hydrogen peroxide disappearance. However, whatever effect if any, this may have on thymidine incorporation (Figure 6.8) cannot be deduced from the results shown here.

NAC, 0.1mM, did not reduce the DCF fluorescence induced by purified T cells stimulated with PMA + anti-CD3. The concentration of NAC was chosen from the "first" inhibitory phase of the concentration-response curve (Figure 6.8) and was effective at promoting 40% inhibition of the control proliferative response.

The results presented in Figure 6.7 and 6.10 showed that vitamin E had no significant effect on the proliferative response, or on ROS formation, in purified T cells stimulated by PMA, 0.3nM, plus anti-CD3. Vitamin E has been reported to inhibit PKC activity (Mahony and Azzi, 1988). It could be that the lack of inhibition by vitamin E on purified T cells is related to a change in the sensitivity of these cells to vitamin E, in the absence and presence of accessory cells. Indeed, not all cells have been shown to be equally sensitive to the effects of vitamin E, and whether this lack of sensitivity in purified T cells is due to a chemical, cellular or biochemical factor, cannot be inferred from the results presented here.

In summary, the experiments conducted in this chapter showed that T cell proliferation was inhibited by a series of compounds known to inhibit free radical-mediated processes. By flow cytometry, using the free radical sensitive dye, 2'7'-dichlorofluorescein, it was shown that the phorbol ester, PMA, can induce

lymphocytes to generate a ROS signal which is independent of accessory or phagocytic cells. By comparing the ROS signal-sensitivities induced by purified T cells and PBMC, to desferrioxamine and DMSO, the results suggest that the nature of the radical species generated by T lymphocytes in the presence and absence of accessory cells differs. Furthermore, it was possible to block proliferation by these hydroxyl radical scavengers while not significantly affecting the total oxygen radical generation. For the above reasons, the results provide evidence compatible with the hypothesis that cellular activation is tightly regulated by the redox status of the cell (Burdon, 1995; Demple *et al.,* 1991) (probably via redox-sensitive proteins), rather than from the direct action of ROS on essential molecular "targets" for T cell signalling. Further studies directed towards identifying the influence of ROS on these redox-sensitive enzymes will help to understand these data.

CHAPTER 7.

ROLE OF REACTIVE OXYGEN SPECIES IN THE "NEGATIVE SIGNAL" PATHWAY ACTIVATED BY ANTI-CD2 MONOCLONAL ANTIBODY OKT11$_1$.

Introduction.

The experiments performed in this chapter were designed to study the role of ROS in the signalling pathway activated by anti-CD2 mAb $OKT11_1$.

CD2 has three distinct epitopes termed $T11_1$, $T11_2$ and $T11_3$. The combination of anti-CD2 molecules used determines whether CD2 functions as an activator or inhibitor of T cell activation. The work performed in this chapter used an anti-CD2 monoclonal antibody directed against the $T11_1$ epitope of the CD2 molecule. It is speculated that the structure detected by the $T11_1$ functions as a "negative signal receptor". However, the exact nature of the biochemical messengers generated following $T11_1$ binding are incompletely understood.

It is possible for anti-CD3 alone to induce a proliferative response in a mixed cell population. This effect can be explained by the "two signal hypothesis" for T cell activation (Lafferty *et al.*, 1985); the "first signal" provided by anti-CD3 and the second by contact with an antigen-presenting cell. The effect of anti-CD2 on anti-CD3-induced proliferation was examined. In order to study whether CD2 exerted its effects on proliferation through ROS generation, cells were treated with the antioxidants, ascorbic acid and N-acetyl cysteine (NAC). Further studies were performed to test anti-CD2 alone, and in combination with anti-CD3, for ROS production, using the free radical sensitive dye 2'7'-dichlorofluorescein.

7.1 Effect of anti-CD2 on anti-CD3 induced proliferation.

PBMC were incubated with a range of anti-CD3 concentrations, 0.001-1μg/ml, in the presence and absence of anti-CD2, 500ng/ml. Figure 7.1 shows that anti-CD2, 500ng/ml, significantly reduced CD3-dependent proliferation.

Anti-CD2, 500ng/ml, alone had no significant effect on thymidine incorporation. Anti-CD3 at concentrations between 0.01 to 0.1μg/ml produced maximum proliferation. Cells stimulated with anti-CD3, 0.01-0.1μg/ml, plus anti-CD2 resulted in the greatest suppression of proliferation (>80%).

From these data concentrations of anti-CD3, 0.05μg/ml, and anti-CD2, 500ng/ml, were used in subsequent experiments.

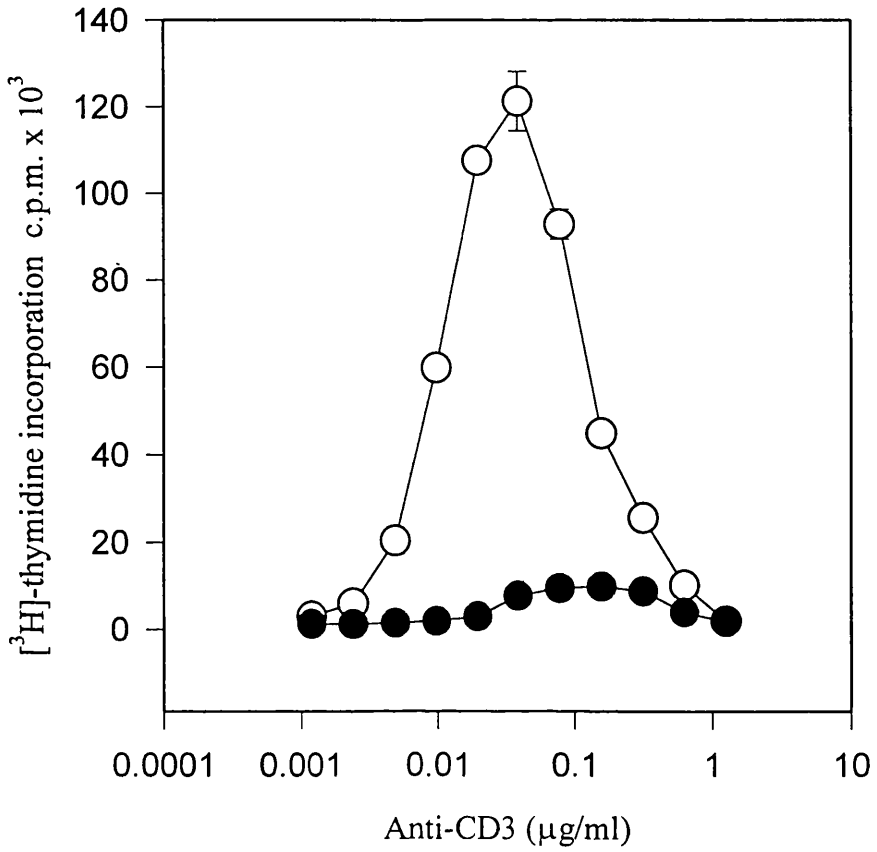


Figure 7.1
The influence of anti-CD2 on the anti-CD3-induced thymidine incorporation.
The effect of anti-CD2 mAb OKT11, 500ng/ml, on proliferation of PBMC stimulated with anti-CD3 mAb $UCHT_1$. Cells were incubated with concentrations of anti-CD3 shown: (O) in the absence of, or (●) in the presence of anti-CD2, 500ng/ml. After 32 hours of culture, the cells were pulsed with [^{3}H]-thymidine and incubated for a further 16 hours. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. The data was statistically analysed using 2-way ANOVAs. The p value obtained for anti-CD3 vs anti-CD3 + anti-CD2 was 0.0097.

The antioxidants, ascorbic acid and NAC, were present throughout the 48 hour incubation period. Control values were obtained from cells stimulated with anti-CD3, 0.05μg/ml, and anti-CD2, 500ng/ml, without antioxidant. Anti-CD2 inhibited CD3-induced proliferation by 83%.

7.2 Effect of ascorbic acid on the proliferative response stimulated by anti-CD3 plus anti-CD2.

Figure 7.2 shows that ascorbic acid, 0.002-5mM, reversed the anti-CD2-induced inhibition of the proliferative response stimulated by anti-CD3. Thymidine incorporation by cells treated with ascorbic acid were statistically significantly different ($p<0.05$) from control. This effect ranged from about 54% above control at 0.002mM to about 82% above control at 1mM.

7.3 Effect of NAC on the proliferative response stimulated by anti-CD3 plus anti-CD2.

Figure 7.3 shows that NAC, at concentrations between 0.2 to 25mM, significantly reversed the anti-CD2-induced inhibition of the CD3-mediated proliferative response. This effect ranged from about 47% above control at 0.2mM to about 80% above control at 12.5mM.

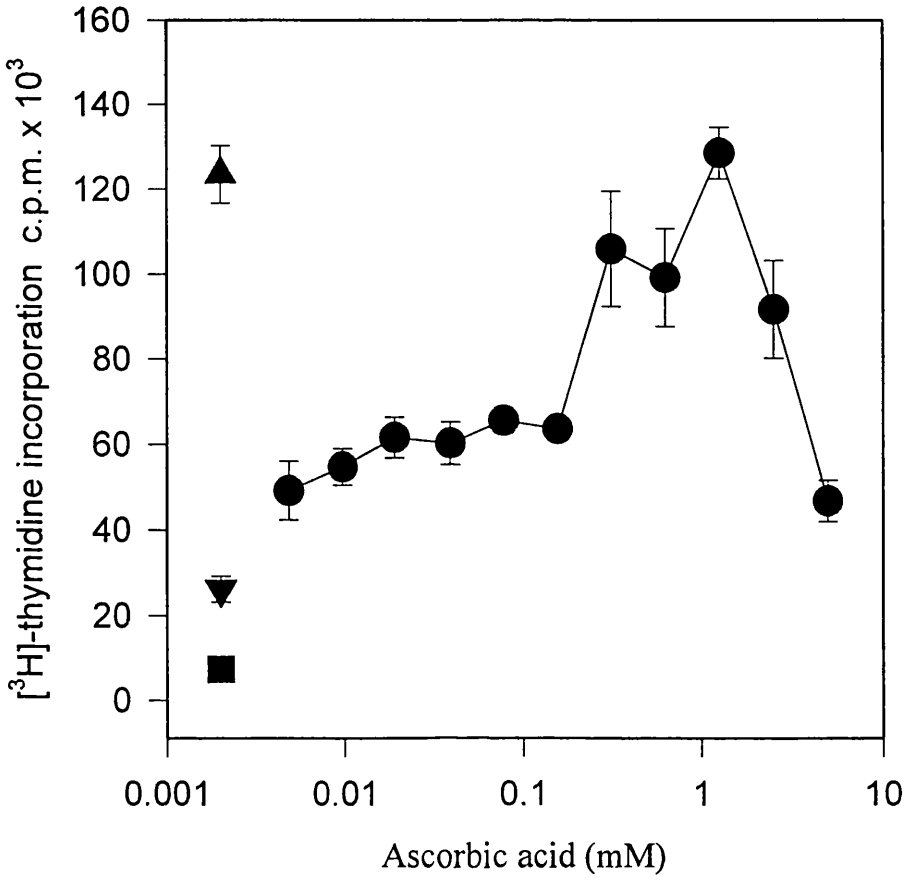


Figure 7.2
The effect of ascorbic acid on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of ascorbic acid on proliferation of PBMC stimulated with anti-CD3 mAb $UCHT_1$, 0.05μg/ml, plus anti-CD2 mAb OKT11, 500ng/ml. Cells were incubated with ascorbic acid at the concentrations shown. Anti-CD3 and anti-CD2 were added simultaneously. Cells were incubated for 48 hours prior to harvesting. ▲ anti-CD3, 0.05μg/ml, alone; ■ anti-CD2, 500ng/ml, alone; ▼ anti-CD3 + anti-CD2; ● anti-CD3 + anti-CD2 + ascorbic acid. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. All points (●) were significantly different ($p<0.05$) from control values in the absence of ascorbic acid (▼), using the Student's t test.

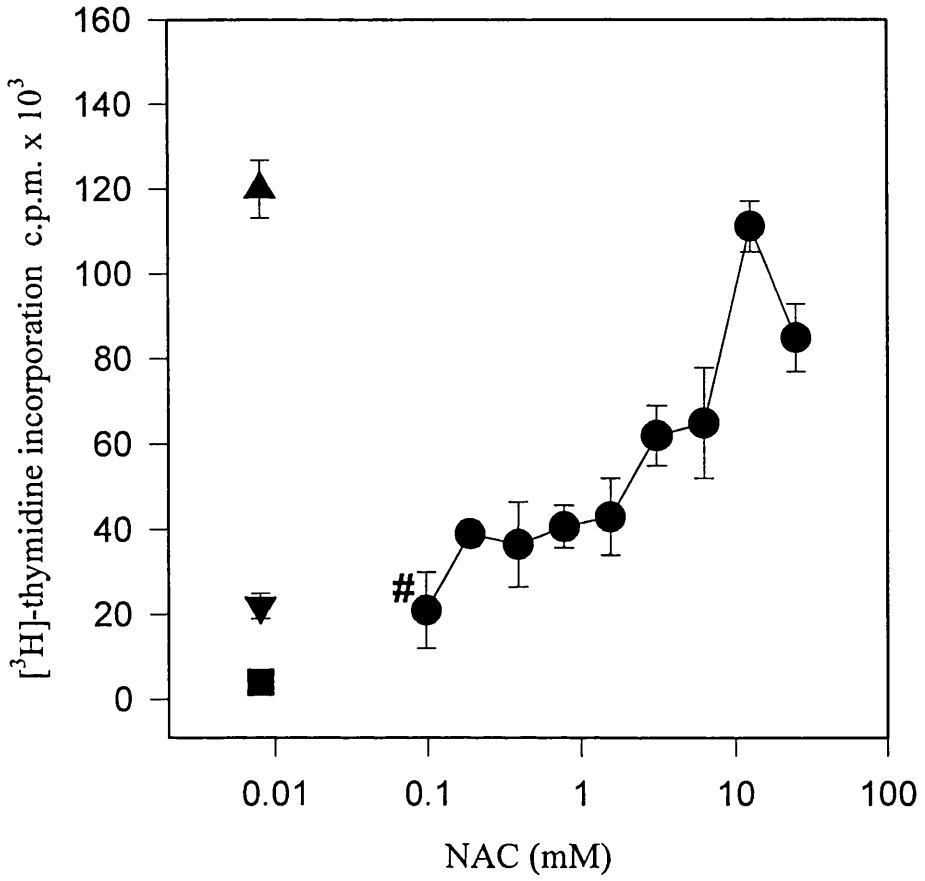


Figure 7.3
The effect of NAC on thymidine incorporation by PBMC.
The concentration-response relationship for the effect of NAC on proliferation of PBMC stimulated with anti-CD3 mAb $UCHT_1$, 0.05μg/ml, plus anti-CD2 mAb OKT11, 500ng/ml. Cells were incubated with NAC at the concentrations shown. Anti-CD3 and anti-CD2 were added simultaneously. Cells were incubated for 48 hours prior to the determination of thymidine incorporation. ▲ anti-CD3, 0.05μg/ml alone; ■ anti-CD2, 500ng/ml alone; ▼ anti-CD3 + anti-CD2; ● anti-CD3 + anti-CD2 + NAC. Each point represents the mean ± s.e.m. of triplicate determinations from three independent experiments. # indicates that a point is not significantly different ($p>0.05$) from control values in the absence of NAC.

7.4 Effect of anti-CD2 mAb $OKT11_1$ on free radical production by PBMC.

Figure 7.4 shows that anti-CD2 alone caused a dose-dependent increase in DCF fluorescence, without the apparent need for a "second" stimulus. The anti-CD2-induced ROS signal was rapid (within 5 minutes) for all concentrations tested; 0.25-1μg/ml. Anti-CD2, 1μg/ml, produced an increase in the DCF fluorescence which was maintained over the 40 minute period. The ROS signal stimulated by anti-CD2, 500ng/ml, reached a maximum at 25 minutes, followed by a steady decline. The maximum mean fluorescence change for anti-CD2, 1μg/ml, 500ng/ml and 250ng/ml was 500, 260 and 100 units respectively.

7.5 Effect of anti-CD3 on anti-CD2-induced free radical production.

Figure 7.5 shows that anti-CD3, 0.05μg/ml, does not produce any significant change ($p>0.05$) in the anti-CD2 (500ng/ml)-induced ROS signal. The profile of the effect of anti-CD2 on ROS generation was no different from that using a combination of anti-CD3 plus anti-CD2 over the entire 40 minute period.

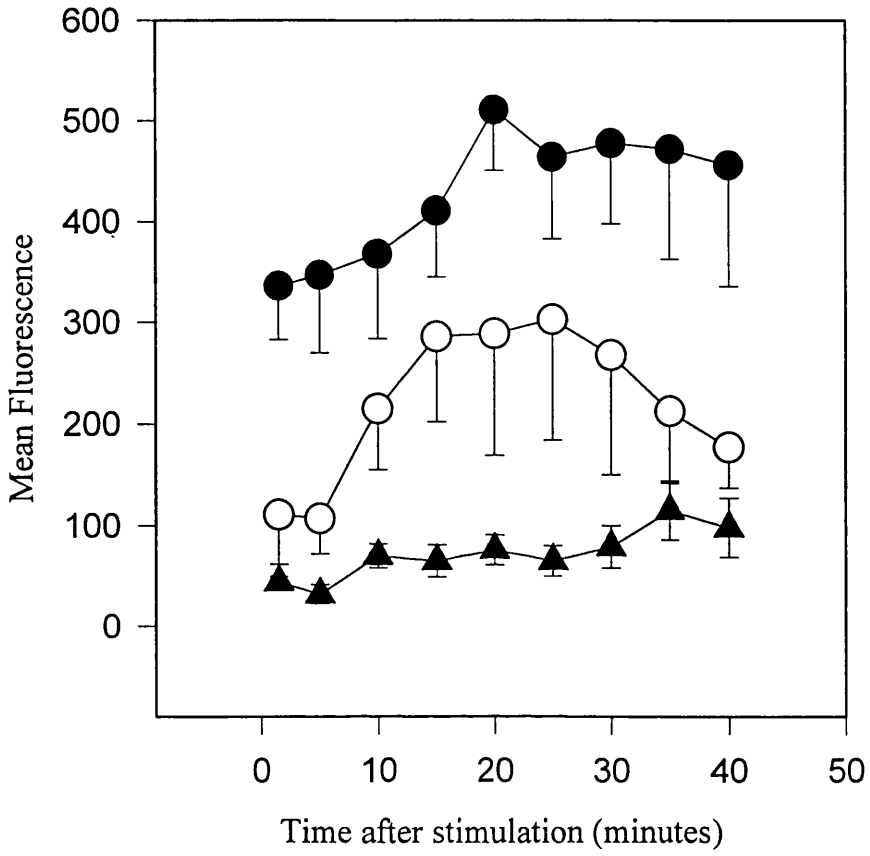


Figure 7.4
The effect of anti-CD2 mAb OKT11$_1$ on the DCF fluorescence of PBL.
The concentration-response relationship for the effect of anti-CD2 mAb OKT11$_1$ on the ROS production by the lymphocyte population from PBMC. Cells were loaded with DCFH-DA, 10μM, 15 minutes prior to the addition of: ▲ anti-CD2, 250ng/ml; ○ anti-CD2, 500ng/ml; ● anti-CD2, 1μg/ml. Mean fluorescence readings were taken every 5 minutes and subtracted from parallel cultures in the absence of anti-CD2 at each time point. The data are the mean ± s.e.m. from three separate experiments. 2-way ANOVAs were used to statistically analyse the data following a 20 minute delay The p values obtained for anti-CD2, 1μg/ml, vs 250ng/ml and 500ng/ml, were both less than 0.0001; p value for 500ng/ml anti-CD2 vs 250ng/ml was 0.0067.

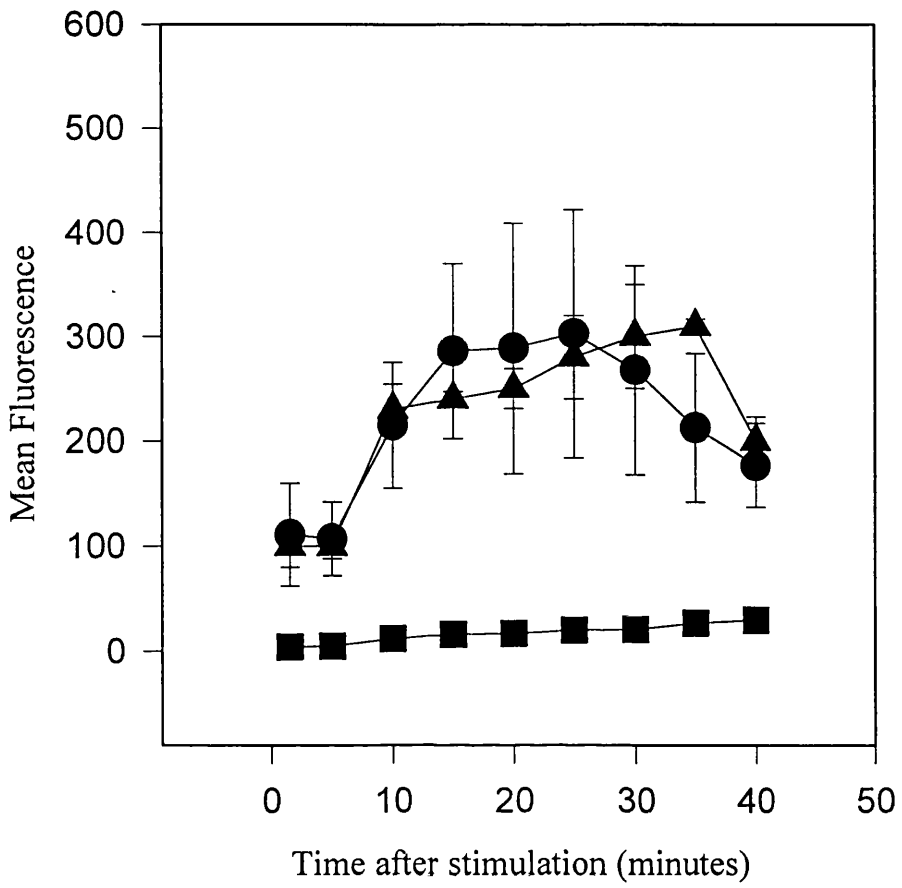


Figure 7.5
The influence of anti-CD2 on the DCF fluorescence from anti-CD3-stimulated PBMC.
The effect of anti-CD2 mAb $OKT11_1$, 500ng/ml, on ROS production by the lymphocyte population from PBMC, stimulated with anti-CD3 mAb $UCHT_1$, 0.05μg/ml. Cells were loaded with DCFH-DA, 10μM, 15 minutes before the addition of stimuli: ● anti-CD2, 500ng/ml, alone; ■ anti-CD3, 0.05μg/ml alone; ▲ anti-CD2 + anti-CD3. Mean fluorescence data was collected every 5 minutes and subtracted from parallel cultures in the absence of anti-CD2 and anti-CD3. The results show the mean ± s.e.m. from three separate experiments.

Discussion.

The experimental aim in this last chapter was to examine the role of anti-CD2-induced ROS production in T cell activation.

The anti-CD2 mAb OKT11_1 was used; it recognises one of three distinct epitopes ($T11_1$, $T11_2$, $T11_3$) termed $T11_1$. CD2 interacts with its physiological counter receptor, LFA-3 (CD58), which has a broad tissue distribution (including monocytes). Like anti-CD3 antibodies, anti-CD2 reactivity is restricted to the lymphocyte lineage. The CD2-activated pathway is not physically linked to the CD3-receptor complex and is thought to represent an antigen independent "alternative" pathway of T cell activation (Meuer *et al.,* 1984). The experiments covered in this chapter investigated the interactions between the antigen specific (CD3) and alternative pathway (CD2) of activation.

It is generally accepted that CD3 antibodies interact with "activation receptors" on T lymphocytes. Human T lymphocytes are usually defined and isolated by their unique property of spontaneously forming rosettes with sheep erythrocytes (SRBC). Monoclonal antibodies against receptors for SRBC on T lymphocytes have been shown to identify a polypeptide of molecular weight 50 KDa (Kamoun *et al.,* 1981). Much controversy exists concerning the nature of the epitope associated with rosetting, but it is speculated that it may transmit a negative signal for T lymphocyte activation. The biochemical nature of this signal is unknown.

The results presented in Figure 7.1 are in agreement with other reports (Palacious *et al.,* 1982; Van Wauve *et al.,* 1981; Ohno *et al.,* 1991) which show that CD3-stimulated cells from cultures exposed to anti-CD2, 500ng/ml, proliferated significantly less than cells from cultures in the absence of anti-CD2. Anti-CD3 mAb is mitogenic for T cells only in the presence of antigen-presenting cells (as shown in chapters 4 and 6). Anti-CD2 mAb OKT11_1 was not mitogenic for T cells when used alone. If CD2 was interfering with the delivery of the CD3 signal, one may expect that at a fixed concentration of anti-CD2, the inhibitory response could be overcome by increasing the concentration of anti-CD3. However, the inhibitory activity of anti-

CD2 mAb $OKT11_1$ was observed over a wide range of the anti-CD3 concentrations used, and could not be overcome by simply increasing the anti-CD3 concentration.

Flow cytometry experiments were performed in an attempt to define further the mechanism whereby CD2 inhibits anti-CD3-induced proliferation (Figure 7.4). These investigations contributed to perhaps the most interesting and important findings, showing that anti-CD2 alone could generate a dose-dependent rapid and sustained ROS signal. In addition, there was no lag period (15-20 minutes) as seen with PMA-induced ROS generation. These data (and those using anti-CD28 mAb 15E9 in chapter 5) are at odds with a previous study by Benichou *et al.,* (1989) which showed that antibodies, namely anti-CD2 and anti-CD28, were unable to produce an oxygen radical signal. A number of experimental differences between the study of Benichou *et al.,* (1989) and this study could explain the discrepancy. Firstly, in their study, chemiluminescence was used to assess ROS formation, and a Jurkat tumour T cell line was used. In addition, the authors stated that the Jurkat cell line used needed modification since it was heterogeneous, ie. certain subsets being able to produce ROS while others were not. It is generally agreed that Jurkat cells are not truly representative of human T cells, and may differ in their signalling mechanisms and cell structures (CD2 and CD28) when compared to normal human T cells, therefore being a poor study tool. Secondly, (as shown in chapter 5 using anti-CD28) the type of anti-CD2 and anti-CD28 mAbs tested were not cited by the authors. It would not be surprising if like anti-CD28 mAb, there are different anti-CD2 mAbs which recognise distinct epitopes, each of which differ in their ability to generate ROS. Further studies to determine the nature of the epitope recognised by "ROS-producing" anti-CD2 mAbs would help to clarify this point.

The finding that the anti-CD2-mediated "inhibitory mechanism" could be partly reversed by the antioxidants ascorbic acid and NAC suggests that ROS could be important in this mechanism (Figures 7.2, 7.3). The incomplete reversal of inhibition by the antioxidants could be related to the involvement of a number of different, independent pathways. Assuming that not all of these pathways are sensitive to antioxidants may explain why there is only a partial reversal of the "anti-

proliferative" effect of anti-CD2. The results presented here, however, do not allow a deduction of the exact involvement of ROS in the anti-CD2-induced inhibition, or of the exact cellular site of action of these antioxidant compounds. Indeed, it is possible that the antioxidants exert effects on proliferation additional to their antioxidant properties, as discussed in chapter 4.

The results presented in Figure 7.5 showed that anti-CD3 did not interfere with the ROS signal produced by anti-CD2. This excluded the possibility that inhibition of proliferation was directly related to an excess ROS generation, which may have inhibited cellular responses by a cytoxic action (as discussed in section 1.3). However, others have suggested that the ROS generated could be down-regulating T cell activation and contributing to T cell anergy (Buttke *et al.,* 1994; Sarafian *et al.,* 1994).

The contribution of other accessory/adhesion molecules in the anti-CD2-induced ROS signal could not be deduced from this system (compared to the purified T cell system used in chapter 6). However, a recent study using murine thymocytes has demonstrated that the negative regulation of antigen-specific T cell activation by the CD2-mediated signal is antigen-independent (Ohno *et al.,* 1991).

To date, evidence suggests that CD2 may mediate a separate pathway of T cell activation, possibly through cyclic AMP or the CD45 phosphatase. As mentioned earlier (section 1.2.6.B), cyclic AMP is considered to be a second messenger responsible for negative regulation of T cell responses. LFA-3 binding has shown to increase the cyclic AMP levels in human T cells (Carrera *et al.,* 1988). In addition CD2 ligation has been shown to alter the phosphatase activity of CD45 (Schraven *et al.,* 1990) resulting in negative regulation of the activation signal through the T cell receptor-CD3 complex. Whether cyclic AMP- or CD45-mediated processes are influenced by ROS, cannot be inferred from these data alone.

Since the effect of anti-CD2 on anti-CD3-induced IL-2 release was not determined, any contribution anti-CD2 may have made to the early T cell activation events cannot be deduced. To understand the mechanism of action of anti-CD2-induced ROS production, a number of questions, including the following, remain to be answered:

(i) Do the antioxidants tested here, also inhibit the anti-CD2-induced ROS signal? A similar protocol, to that followed in chapter 4 could be used.

(ii) Does anti-CD2 inhibit CD3-mediated IL-2 release in a similar manner to that seen in the proliferative assay? The supernatants could be aspirated following the 48 hour incubation period with anti-CD3 plus anti-CD2. CTLL could be used to determine the IL-2 release in the culture samples, as discussed in previous chapters.

(iii) Is the presence of accessory cells essential for anti-CD2 stimulated ROS production?

(iv) If anti-CD2 does produce a ROS signal in purified T cells, does the nature of the ROS signal change, as seen with the PMA-induced free radical production in chapter 4? One approach to examine this, would be to test the sensitivity of the anti-CD2-induced ROS signal, in the presence and absence of accessory cells, to hydroxyl radical scavengers, as discussed in chapter 6.

(v) Does anti-CD2 influence T cell activation indirectly through alterations in the cellular redox status? One approach to address this final question would be to determine the influence of anti-CD2 on intracellular glutathione levels. This could be done using flow cytometry, making use of the dye monochlorobimane (June *et al.*, 1992).

In conclusion, the results in this chapter suggest that anti-CD2 mAb $OKT11_1$ reacts with a T cell component that is associated with the inhibitor of proliferation signalling. CD2 ligation was shown to induce a powerful ROS signal. The ROS generated through anti-CD2 binding may therefore influence the many processes of T cell stimulation, directly or indirectly, through modulation of the redox status of the cell.

CHAPTER 8.

GENERAL DISCUSSION.

General discussion.

The overall impression one obtains from this experimental work, and a review of other studies of the effects of ROS and antioxidant compounds on various lymphocyte functions, is that ROS appear to influence T cell signalling indirectly, through alterations in the redox state of the cell, rather than acting on specific "oxidative-stress receptors".

Since the studies in this thesis were performed with various mitogens (phorbol ester, PMA; calcium ionophore, A23187; monoclonal antibodies (mAb) against CD3, CD2, CD28) that were likely to affect different cell subpopulations, the experiments performed in chapters 3, 4, 5 and 7 were conducted using unseparated human peripheral blood mononuclear cells (PBMC). This provided culture conditions that more closely matched those of the in vivo environment. The results from chapter 6 were obtained from using purified T cells in order to eliminate the possibility that ROS detected from the lymphocyte population in a mixed population was influenced by contaminating phagocytes.

The results presented in chapters 3 and 4 demonstrated that the synergism between a phorbol ester, PMA, and either a calcium ionophore, A23187, or anti-CD3, for lymphocyte proliferation and IL-2 release, is accompanied by an increase in ROS formation. Chapter 5 showed that it was possible to increase proliferation and IL-2 release without an increase in free radical formation (anti-CD28 in combination with either, PMA or anti-CD3), implying that ROS formation was not the sole pathway of transmembrane signal transduction. A more likely proposition is that ROS formation is a coordinate and necessary biochemical event that relates indirectly to cell proliferation and IL-2 release, via its influence on cellular redox status. This is supported by the results in chapter 4 which showed that it was possible to block ROS generation by hydroxyl radical scavengers but still allow a proliferative response. In addition, the concentration of antioxidant needed to inhibit proliferation and IL-2 release by 50% of its control value was often several orders of magnitude higher than that needed to inhibit ROS generation by a similar degree.

In chapter 6, it was demonstrated that the phorbol ester, PMA, can stimulate lymphocytes to produce a ROS signal which is independent of accessory cells. The nature of this ROS signal appeared to be different to that observed in a mixed cell population, as demonstrated by the lack of effect of hydroxyl radical scavengers on the DCF fluorescence of purified T cells. However, these results do not allow a deduction of the site of action, or source of ROS, in the T cell.

To extend the studies presented here, and increase understanding of ROS influence in T cell activation, the following questions still need to be answered:

(i) How do different stimuli, such as PMA, A23187, mAbs against CD3, CD28 and CD2, generate differing amounts of ROS fluxes, as assessed by the change in DCF fluorescence. One approach to address this question would be to examine the effects of these stimuli on possible sources of ROS in lymphocytes. For example arachidonic acid metabolism and the NADPH-oxidase like system. This point will be taken up again later in this chapter.

(ii) What are the effects of ROS on the cellular glutathione levels? The work here has suggested that ROS are modulators of the redox state of the cell, influencing cellular signalling indirectly. Glutathione is the major regulator of the redox state. Consequently, alterations in the glutathione content of the cell would influence many redox-sensitive proteins, such as, protein kinases, transcription factors or transcription factor inhibitors (eg. IκB). A more advanced technique to measure intracellular glutathione would be to use flow cytometry making use of the dye monochlorobimane.

The work presented in this thesis has shown that many different T cell stimulants are capable of activating some unknown mechanism of ROS generation. This effect had a variable range: (i) a mean DCF fluorescence change of less than 100 units stimulated with anti-CD3 (chapter 4) or anti-CD28 mAb 913.12 (chapter 5); (ii) mean fluorescence change between 200-600 units stimulated by PMA (chapters 3-6), or, anti-CD28 mAb 15E9 (chapter 5), or, anti-CD2 mAb $OKT11_1$ (chapter 7); (iii) mean fluorescence change greater than 1000 units stimulated by a combination of PMA + A23187 (chapter 3), or, PMA + anti-CD3 (chapter 4).

These data implied that maybe there was more than one source of ROS in lymphocytes, and that differing radical species can be generated. Below is an attempt to explain the possible sources of ROS in T lymphocyte activation, with particular emphasis to how the results from this thesis confirm some of the proposed sources of ROS.

Its important to note that the technique used in our study is indicative of an intracellular oxidative process, but cannot predict the mechanism implicated in this oxidation. It is unfortunate that the ROS assays are unable to distinguish between the production of superoxide radical anions and hydroxyl radicals. In addition, the effects of the antioxidants are not specific to ROS. To date, the tools available for ROS study are still primitive. Previous studies have related it to the production of hydrogen peroxide (Roth and Valet, 1990), but it could also result from the hydroxyl radical, superoxide anion radical, or from a radical originating from lipid peroxidation.
The results presented in this thesis suggest that more than one source of ROS generation is likely. Indeed, several enzymes and intracellular electron transfer reactions are known to produce ROS which may be involved in intracellular signalling (Halliwell and Gutteridge, 1990). The most important of these being the membrane bound NADPH oxidase, and the enzymes induced in the metabolism of arachidonic acid, namely cyclooxygenase and lipoxygenase. Data from this thesis does not resolve which of these two processes are involved in ROS production, but its interesting to speculate about a model for free radical generation. Firstly, a brief introduction to the NADPH oxidase system will be presented followed by data from the thesis which support the role of this enzyme in T cell ROS generation. A similar process will be followed for the enzymes involved in arachidonic acid metabolism.

Exposure of human neutrophils to a variety of soluble or particulate stimuli results in activation of the respiratory burst during which ROS, such as superoxide anion radicals and hydrogen peroxide, are generated. Superoxide anion radicals are converted to hydrogen peroxide (either spontaneously or by superoxide dismutase)

and hydroxyl radicals. Phagocytic cells have been known for some time to possess a special NADPH oxidase ("respiratory burst oxidase") which upon activation, yields superoxide anion radicals by a one electron reduction of dioxygen (Babior, 1988). Whilst the exact nature of NADPH oxidase is unclear, it is thought to be a multicomponent electron chain. Regulation of the NADPH oxidase involves the apparent physical separation of the various protein components of the enzyme system. Of the proteins which have been characterised and cloned, two are integral membrane proteins (includes the cytochome b) and two are cytosolic, identified in fractionated neutrophils. Although phagocytic cells have been known for some time to possess NADPH oxidase, studies showing that some non-phagocytic cells, including B cells (Maly, 1989; 1990), and T cells (Sekkat *et al.,* 1988), are capable of generating ROS, have suggested that these cells may have a NADPH oxidase-like system. To date, T cells have not been shown to contain a NADPH oxidase, but it has been reported that these cells contain the membrane-associated component of the phagocyte specific NADPH oxidase, and that this component can produce ROS when it is activated in the presence of phagocyte cytosol (Pick and Gadba, 1988). However, at the present time it remains unknown if lymphocytes contain the cytosolic factors which are an essential requirement for the activation of the neutrophil oxidase (Jones *et al.,* 1992). Interestingly, PMA has been shown to stimulate phosphorylation of a cytosolic oxidase component, known as p47-phox when neutrophils are stimulated (Heyworth *et al.,* 1992). Since PMA is a protein kinase C activator, this suggests an important role for protein kinase C in superoxide anion radical and hydrogen peroxide generation. However, the exact implications of this finding at present are unclear since superoxide anion radical generation has been demonstrated to occur independent of p47-phox phosphorylation.

The results presented in this study show that PMA can generate a ROS signal in PBMC, but also in purified T cells. Rabesandratana *et al* claimed that purified T cells were unable to generate ROS since they lacked an essential component of the ROS generation pathway and hypothesised that stimulated phagocytes might provide them with this component. To elaborate from this theory, it is possible that PMA activates

two independent ROS pathways, one of which involves a NADPH oxidase-like system. The fact that the nature of the PMA-induced ROS signal changed in purified T cells could be explained by PMA activating two separate and independent mechanisms in the presence and absence of phagocytic cells. Indeed, it could be that in lymphocytes from a mixed cell population, the essential component of the NADPH oxidase system is provided by the phagocytic cells, allowing two ROS pathways to co-exist, one involving a NADPH oxidase (pathway 1) and one not (pathway 2). Thus, it is possible that in purified T lymphocytes another mechanism, independent of NADPH oxidase, exists for ROS generation. This is depicted in Figure 8.1.

Arachidonic acid is metabolised by two distinct oxygenase pathways; a cyclooxgenase catalyses the conversion of arachidonic acid to prostaglandins and related products, while a lipoxygenase coverts arachidonic acid to leukotrienes. Both pathways generate intracellular ROS as by-products. For example, hydroxy-eicosatetranoic acid metabolism in the lipoxygenase pathway generates ROS (Goodman *et al.*, 1980). Thus arachidonic acid metabolism could be the second source of ROS in the lymphocyte. A recent study using anti-CD28 has suggested that lipoxygenases are involved in the anti-CD28 induced ROS formation by lymphocytes (Los *et al.*, 1995).

Since the production of free arachidonic acid is the rate-limiting step in the production of eicosanoids and hence ROS, intracellular mobilization of arachidonic acid in response to exogenous stimuli is a crucial factor. It is well documented that the release of free arachidonic acid from cellular phospholipids is mediated via the action of an intracellular phospholipase A_2 (PLA_2). Mammalian cells contain a PLA_2 which is calcium sensitive and responsive to G-protein activation. The PLA_2 has also been found to be a substrate for PKC (Boyer *et al.*, 1995). Hence PMA could also activate ROS production through PLA_2 activation. Conversely, Haddox and coworkers (1978) have shown that oxidants can increase cellular levels of guanylate cyclase while thiol-containing components decrease their levels. Since cGMP is known to be correlated with arachidonic acid metabolism (Nishizuka, 1986), this

suggests that oxidative processes may be involved in additional signals necessary for T lymphocyte activation, possibly through the arachidonic acid cascade.

In addition, evidence is emerging that the activation of PLA_2 can be regulated by exogenous hydrogen peroxide. Hydrogen peroxide is thought to act as a "messenger", acting not through the more general mechanism of calcium disturbances or lipid peroxidation, but rather indirectly via stimulation of intracellular kinase activity; thus suggesting that PKC is not exclusively responsible for PLA_2 actvity (Boyer *et al.*, 1995). The nature of the tyrosine kinase activated by CD2 is unknown. Hence, it would be interesting to speculate that CD2 may activate a kinase which could then activate PLA_2. Furthermore, it may be that in PBMC, ROS generated through a NADPH oxidase like system could activate PLA_2, allowing both ROS mechanisms to co-exist. This may explain the synergy between PMA plus A23187, and PMA and anti-CD3, for ROS formation, as assessed by an increase in DCF fluorescence. It would be interesting to test anti-CD28 mAb 15E9 (shown to generate ROS in chapter 5) with PMA to provide support for this possibility. Another explanation for the observed synergism between PMA + A23187, and PMA + anti-CD3, for ROS formation, may be related to calcium modulation. It is conceivable that the increase in intracellular calcium triggered by anti-CD3 and produced by A23187 may co-activate PLA_2 and 5-lipoxygenase, which are both calcium-dependent. It has been reported by Ikebuchi *et al.*, (1991) that the activation of PLA_2 requires calcium above a threshold level. In addition, diacylglycerol (DAG) formation through the T-cell receptor-activated phospholipase-$C\gamma_1$ may be converted to arachidonic acid by DAG lipase and thereby redirected to the lipoxygenase pathway.

This potential second pathway for ROS generation is complete in lymphocytes and is independent of accessory cells, thus it could be responsible for the ROS signal generated by purified T cells following PMA stimulation.

The formation of superoxide anion radicals has been implicated in the formation of thromboxanes and prostaglandins (Mittal *et al.*, 1977). Accordingly, the main radical identified in purified T cells was not sensitive to desferrioxamine or DMSO; hence it was thought not to be the hydroxyl radical. However, the ROS signal generated by

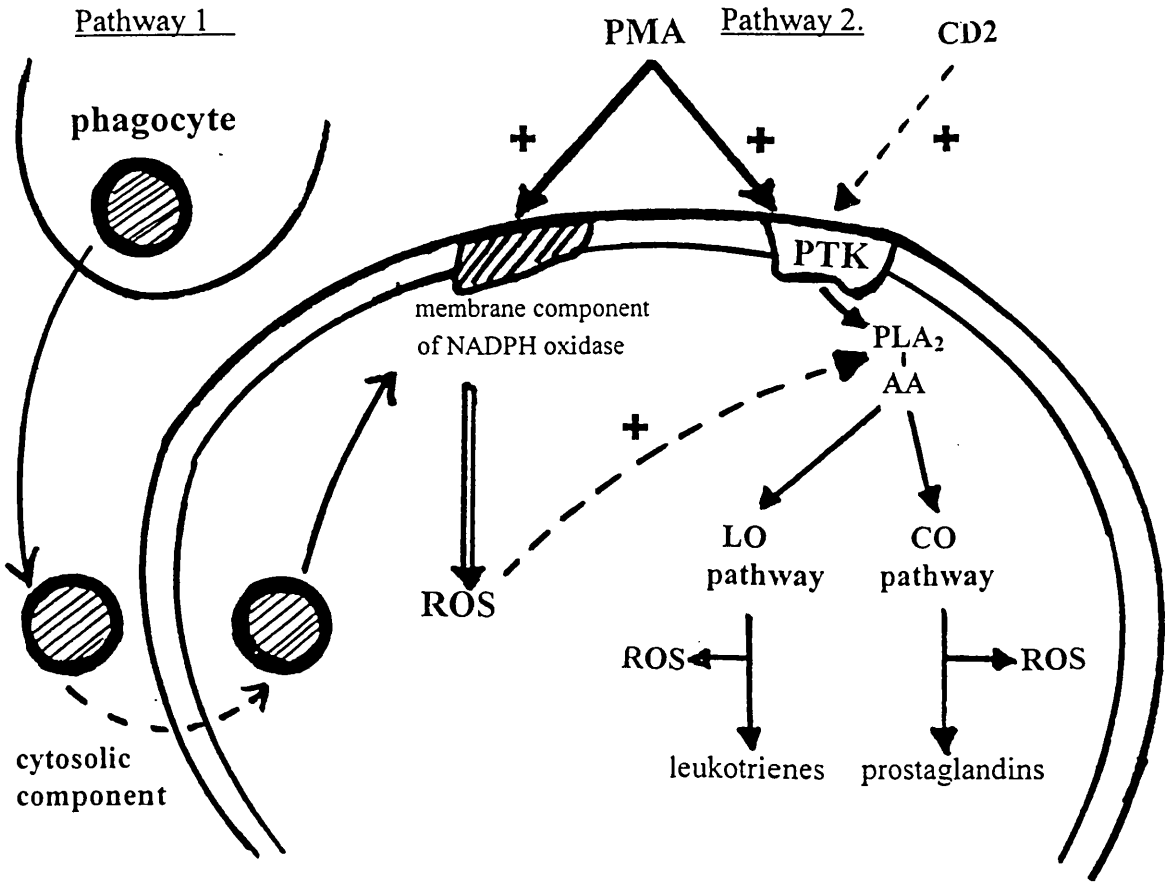
purified T cells was effectively blocked by ascorbic acid, known to react with superoxide anion radicals (Niki, 1991). This is shown in Figure 8.1.

Further studies directed towards identifying the "proposed source" of ROS following stimulation with the many stimuli used in the experiments performed in this thesis will help to clarify some of the above points. An interesting set of experiments to include in future investigations would be to use cells from patients with Chronic Granulomatous Disease (CGD). These patients are thought to lack an essential component of the NADPH oxidase system, cytochrome b-like component. Consequently, they do not generate any superoxide anion radicals or hydrogen peroxide. If the assumptions outlined in Figure 8.1 are correct, then PMA stimulation would produce a diminished ROS signal (or perhaps even no signal) by lymphocytes from a mixed population (T cells plus phagocytic cells). In addition, the PMA-induced ROS signal by purified T cells from CGD patients should remain unchanged. The involvement of the NADPH oxidase system in free radical production stimulated by anti-CD2 and anti-CD28 mAb 15E9 could also be examined.

One approach to test if arachidonic acid metabolism was responsible for generating free radicals in T cells, would be to use specific lipoxygenase and cycloxygenase inhibitors. Two recently identified specific 5-lipoxygenase inhibitors include MK886 and ICI 230487. This set of experiments would help to define the nature of the ROS signal generated by purified T cells. In addition, arachidonic acid release could be directly measured using radiolabelled arachidonic acid. The release of arachidonic acid following stimulation with a specific activation model could be determined by liquid scintillation counting, thus defining the nature of the source of ROS in lymphocytes, in the presence and absence of accessory cells.

Figure 8.1.

Proposed model for two independent sources of ROS in T lymphocytes.



PBMC only

PBMC & purified T cells

Key

LO lipoxygenase

CO cyclooxygenase

AA arachidonic acid

PTK protein tyrosine kinase

PLA_2 phospholipase A_2

ROS reactive oxygen species

While evidence is emerging to suggest a role for oxidative signalling in T lymphocytes during mitogenic activation, little information is available regarding the mechanism by which this type of signalling exerts its effect, or regarding the interactions that may occur between oxidative signalling processes and the more well described signalling mechanisms that operate during cell cycle entry.

The identification of regulatory proteins such as transcription factors, AP-1 and NF-κB, as molecular targets for oxidative signalling, has been well studied (Schreck *et al.,* 1992; Pahl and Baeuerle, 1995). Evidence cited for and against a role for these transcription factors as molecular "targets" of ROS was discussed previously. Recently, the role of *ras* as a more general target for ROS has been investigated (Lander *et al.,* 1995). This low molecular G protein has attracted great interest in the regulation of T cell activation, although the structure of novel molecules in its pathway remain to be determined (Izuierdo *et al.,* 1995). In T cells, there are indications that at least two mechanisms for *ras* regulation co-exist; one that is PKC mediated and one that is not. The exact molecular details of the latter are unknown but are thought to involve tyrosine kinases (Downward *et al.,*1992).

Since PMA (PKC activator) was the only ROS activator in purified T cells, it could be that ROS production in purified T cells regulates one of the two proposed mechanisms of *ras* regulation, ultimately resulting in a proliferative response. Furthermore, the finding that anti-CD2 alone could produce a ROS signal is in accordance with the discovery that the CD2 antigen can mediate *ras* regulation (Graves *et al.,* 1991). However, much work needs to be done in this field to identify the involvement, if any, of *ras* in the regulation of oxidative-mediated signalling. One approach to test the activation state of *ras* would be to measure the GTP/GDP ratio of immunoprecipitated *ras* following stimulation with phorbol esters, calcium ionophores and the monoclonal antibodies used in this study. A comparison of the amount of activated *ras* to the ROS signal, as determined by an increase in DCF fluorescence, would ascertain the involvement of this G protein in free radical signalling following stimulation with each particular activation model.

In conclusion, the field of future studies involving oxidative-mediated signalling is vast. It seems that critical balances exist in relation to the redox state of the cell and cell proliferation, and while ROS in excess results ultimately in cell death, smaller fluxes may contribute to T cell effector functions indirectly. Further investigations will help to confirm or disprove hypotheses linking the redox status to the overall process of signal transduction.

CHAPTER 9

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ERRATA

Page 30, line 12. Delete 'group'. Insert 'groups'.
Page 31, lines 2 & 20. Delete 'Peri *et al.*'. Insert 'Peri and Veillette'.
Page 32. Reference 'Samelson, L.E., Fletcher, M.C., Ledbetter, J.A., June, C.H. (1990). Activation of tyrosine phosphorylation in human T cells via the CD2 pathway. Regulation by the CD45 tyrosine phosphatase. *J. Immunol.*, **145**: 2448-2454.
Page 48, line 3. Delete 'Izquierdo *et al.*'. Insert 'Izquierdo & Cantrell'.
Page 49, line 6. Delete 'upto'. Insert 'up to'.
Page 60, lines 12, 14, 16 & 29. CD28 should read anti-CD28.
Page 60, line 17. Delete 'aswell'. Insert 'as well'.
Page 104, line 2. Delete 'petri'. Insert 'Petri'.
Page 116, line 20. Delete 'lympocyte'. Insert 'lymphocyte'.
Page 128, line 7. Delete 'stiimulated'. Insert 'stimulated'.
Page 131, line 9. Delete 'intracellulary'. Insert 'intracellularly'.
Page 261, line 13. Delete 'bu not'. Insert 'but not'.
Page 192, line 30. Delete 'A2387'. Insert 'A23187'.
Page 197, line 7. Delete 'dynabead'. Insert 'Dynabead'.
Page 213, line 21. Delete 'was no'. Insert 'were no'.
Page 214, line 2. Delete 'Il-2'. Insert 'IL-2'.
Page 214, line 5. Delete 'atleast'. Insert 'at least'.
Page 219, line 15. Delete 'NACs'. Insert 'NAC's'.
Page 233, line 9. Delete 'cytoxic'. Insert 'cytotoxic'.
Page 239, line 27. Delete 'Rabesandratana *et al.*'. Insert 'Rabesandratana *et al.*, 1992'.
Page 240, line 12. Delete 'coverts'. Insert 'converts'.